Immune response to *Clostridium difficile* infection and an investigation of the mechanisms of moxifloxacin resistance in clinical *C. difficile* isolates

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Declaration

The author performed all the investigations and procedures presented in this thesis, unless otherwise stated.

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Finally, if I am ever heard to express an interest in pursuing another qualification would somebody please just shoot me.

Abstract

Clostridium difficile is an increasingly common cause of nosocomial infection. *C. difficile* infection (CDI) presents as a spectrum ranging from asymptomatic carriage to mild diarrhoea, pseudomembranous colitis, toxic megacolon and intestinal perforation. It is not yet fully understood why this spectrum is seen, however, it is believed that the immune response mounted by an individual plays an important role in determining the outcome of infection.

This thesis comprises three studies. Firstly, a comparative study of immune cell populations within the lamina propria of colonic tissue not exhibiting pathological changes and taken from individuals with symptomatic CDI (cases); asymptomatic carriers; and non-colonised controls. Effector T cells, B cells, plasma cells and macrophages were enumerated by means of immunohistochemical staining of tissue sections. Secondly, a study to establish the prevalence within these three study groups of specific host single nucleotide polymorphisms (SNPs) in the TLR2, TLR5 and IL-8 genes by PCR genotyping and to determine whether an association existed between these genotypes and susceptibility to CDI. Thirdly, an examination of the mechanisms of moxifloxacin resistance in a collection of clinical isolates. This study also sought to determine whether the competitive advantage conferred by resistance to moxifloxacin influenced the fitness of *C. difficile* isolates, in particular growth and the expression of the virulence factors toxins A and B.

Carriers were found to have fewer of all four immune cell types quantified than both cases and controls. However, in only one instance, that of plasma cells, was this

difference statistically significant. Cases had fewer of all cell types than controls but these differences were not significant. These findings suggest that individuals who become infected, both symptomatically and asymptomatically, with *C. difficile* display altered mucosal immune cell populations when compared with those of uninfected individuals.

The data regarding host polymorphisms are suggestive of an association between the presence of SNPs and increased susceptibility to CDI. The variant IL-8 and TLR2 genotypes were carried by cases and carriers while the variant TLR5 genotype was carried by cases only. No variant genotypes were present in control subjects.

All moxifloxacin resistant isolates characterised in this study, with the exception of an isolate with intermediate resistance and a third-generation mutant with reduced susceptibility, carried the common *gyrA* mutation ACT→ATT (Thr82→Ile). Efflux pumps are known to play a role in multi-drug resistance in many bacterial species. Semi-quantitative PCR analysis of expression of the putative efflux pumps *cme* and *cdeA* found no correlation between overexpression and moxifloxacin resistance, suggesting that these genes do not play a role. Three novel mutations in the putative promoter region of CD3197, a MerR family transcriptional regulator found immediately upstream of *cme*, were identified. No association between the presence of these mutations and overexpression of *cme* or resistance or sensitivity to moxifloxacin was found. The competitive advantage conferred by resistance to moxifloxacin does not influence the fitness of *C. difficile* isolates, as measured in terms of growth and toxin production.

Publications

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1. Introduction

1.1 The microorganism

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacillus with a characteristic motility. Cultures have a characteristic odour, often described as resembling that of a barn yard or horse stable, and colonies exhibit chartreuse fluorescence when viewed under long wave UV light. The organism was first isolated in 1935 from the commensal flora of neonates by Hall & O'Toole, who named it *Bacillus difficilis* reflecting the difficulties they had met in the process. They demonstrated that the organism was highly pathogenic in rabbits and guinea pigs due to its secretion of a soluble exotoxin. It was not until the 1970s, however, that an association with human disease was established.

In 1974 Tedesco *et al.,* evaluated 200 patients treated with clindamycin, 10% of whom were found to have a life-threatening condition known as pseudomembranous colitis (PMC) on endoscopy. This was of great concern to the medical profession as clindamycin was widely used to treat anaerobic infections. No pathogens were isolated from the plaque-like lesions seen in the colon. The first observation of a cytopathic toxin in the faeces of patients with PMC was reported by Larson *et al.,* in 1977; in the same year Rifkin *et al.,* demonstrated that this cytopathic effect could be neutralised by *Clostridium sordellii* antitoxin. The following year *C. difficile* was determined to be an aetiological agent of PMC (Bartlett *et al.,* 1978; George *et al.,* 1978; Larson *et al.,* 1978). Today *C. difficile* is the most common cause of nosocomial diarrhoea; it is widely thought to be the aetiological agent of some

15-20% of cases of antibiotic-associated diarrhoea and almost all cases of PMC (Bartlett, 2002).

1.2 Typing of isolates

The ability to type isolates is crucial to an understanding of the epidemiology of any bacterial species and a number of techniques have been developed to type *C. difficile,* recently reviewed by Brazier (2001). Early techniques exploited phenotypic characteristics; however, since the 1980s the predominant methods have been genotype-based.

1.2.1 Phenotypic methods

The earliest methods were based on resistance patterns to antibiotics (Burdon, 1982), analysis of plasmids and soluble proteins (Wüst *et al.,* 1982) and bacteriocin and bacteriophage typing methods (Sell *et al.,* 1983). Immunochemical fingerprinting of EDTA-extracted cell surface proteins has also been used in outbreak studies (Poxton *et al.,* 1984; Sharp & Poxton, 1985).

Delmée *et al.,* (1985) developed a serotyping scheme, based on the earlier work of Nakamura *et al.,* (1981), able to distinguish 19 separate sero-groups. This method is still used as a reference for the comparison of other typing methods (Brazier, 2001).

A novel method of typing, based on the variability in molecular mass of the high and low molecular weight surface layer (S-layer) proteins, has been developed in Edinburgh (McCoubrey & Poxton, 2001; McCoubrey *et al.,* 2003).

1.2.2 Molecular methods

Restriction enzyme analysis (REA)

This method of typing involves the digestion of genomic DNA using the restriction enzyme *Hind*III, a frequent cutter (Clabots *et al.,* 1993). The resulting bands are visualised in an agarose gel and compared by eye to existing exemplars. This method is highly reproducible and extremely discriminatory but time consuming and technically demanding (Brazier, 2001).

Pulsed- field gel electrophoresis (PFGE)

PFGE is another typing method that analyses genomic DNA by means of restriction digest. In this instance rare cutting enzymes, such as *Sma*I, are used. While this method is also highly discriminatory it is time consuming, costly and hindered in certain cases by DNA degradation (Brazier, 2001). However, Fawley & Wilcox (2002) report that the use of thiourea in both the agarose gel and electrophoresis buffer reduces the problem of degradation.

Multilocus variable-number tandem-repeat analysis (MLVA)

This is a recently developed PCR-based technique that amplifies seven regions featuring short tandem repeats distributed over the genome. Isolates are distinguished by analysis of repeat numbers (van den Berg *et al.,* 2007). This method has been shown to differentiate serotypes, toxinotypes and ribotypes and their subtypes and has proven useful in outbreak situations.

Surface layer protein A gene sequence typing (slpAST)

The *slpA* gene encodes the precursor of the S-layer proteins. Variable regions of the gene are amplified by PCR and analysed by way of restriction digest and DNA sequencing (Karjalainen *et al.,* 2001). This method has been shown to discriminate types and subtypes. One advantage of such sequence-based techniques is the portability of the data (Killgore *et al.,* 2008).

Ribotyping

It was reported in 1993 (Gürtler, cited in Cartwright *et al.,* 1995) that the genome of *C. difficile* carried 14 copies of the rRNA gene, each with differing length intergenic spacer regions. Gürtler further demonstrated that variable numbers of different alleles were found in epidemiologically unrelated isolates. PCR amplification of the intergenic spacers generates differing numbers of fragments of variable size; these patterns permit so-called PCR ribotypes to be distinguished one from the other (Cartwright *et al.,* 1995). Ribotyping is the typing method of choice in UK Anaerobe Reference laboratories; the approach currently used was adapted by O'Neill *et al.,* (1996) from that devised by Cartwright *et al.,* (1995). This technique is quick and discriminatory, at least at the ribotype level; subtyping of ribotypes requires the use of a more sensitive method (Brazier, 2001).

Toxinotyping

Toxinotyping was developed by Rupnik *et al.,* (1998). It is premised on the existence of polymorphisms within the genes encoding toxins A and B. These polymorphisms are distinguished by PCR amplification of sections of each gene; the resulting

fragments are digested by restriction enzymes and the length of the products analysed by electrophoresis. While there is good correlation between this method and ribotyping, the latter is more discriminatory (Brazier, 2001).

1.3 The ecology of C. difficile

The spores of *C. difficile* are widely distributed in both the natural and built environment. *C. difficile* has been isolated from sea, river, lake and swimming pool water, soil samples and the surface of raw vegetables (al Saif & Brazier, 1996). Studies have shown that spores are found throughout hospitals and nursing homes; amongst other areas organisms have been isolated from toilet seats, bedpans, floors, bedsheets, call buttons and feeding tube equipment (Cohen *et al.,* 2000a; Fordtran, 2006; Gerding *et al.,* 1995) and portable commode chairs (McFarland *et al.,* 1989). It has been demonstrated that increased levels of environmental contamination are associated with increased prevalence of hand carriage among health care workers, thus the hands of medical personnel can also be sites of contamination and potential sources of cross-transmission (McFarland *et al.,* 1989; Samore *et al.,* 1996).

1.3.1 Food animals

C. difficile colitis is now a common diagnosis in neonatal pigs (Songer, 2004) both in the USA and elsewhere. *C. difficile* has been associated with diarrhoea in calves but no causal relationship has yet been established (Rodriguez-Palacios *et al.,* 2007b). The predominant toxinotype in both pigs and calves is toxinotype V, which is now being seen more frequently in human cases of *C. difficile* infection (CDI). In a three year period between February 2005 and February 2008 the incidence of ribotype 078,

toxinotype V organisms among clinical isolates in the Netherlands increased from 3% to 13% (Goorhuis *et al.,* 2008). Molecular analysis of human and porcine isolates revealed four clonal complexes containing both types of isolates. Similar findings were reported by Debast *et al.,* (2009) and Jhung *et al.,* (2008). Debast *et al.,* concluded that the phenotypic and genotypic characteristics of ribotype 78, toxinotype V isolates from diarrhoeal pigs and human patients in the Netherlands were indistinguishable. In the study by Jhung *et al.,* a high degree of molecular similarity between human and animal toxinotype V isolates was demonstrated. These findings suggest a common origin of human and animal strains or the possibility of transmission between species. Further evidence of this possible route of transmission is provided by the isolation of *C. difficile* from retail meat products; in many instances these isolates have been toxigenic (Rodriguez-Palacios *et al.,* 2007a, 2009; Songer *et al.*, 2009).

1.3.2 Domestic pets

Carriage rates of 23% in household pets (Borriello *et al.,* 1983) and 39.5% among dogs and cats attending a veterinary clinic (Riley *et al.,* 1991) suggest that pets may potentially be a reservoir of infection. Both studies isolated toxigenic and non-toxigenic isolates. However, molecular analysis of animal and human isolates by O'Neill *et al.,* (1993) found no correlation between pet isolates and those found in humans and the hospital environment.

1.3.3 Neonates and children

Carriage rates in healthy adults are typically 3 - 7% (Kato *et al.,* 2001; Kelly & LaMont, 1998). By contrast carriage rates among neonates and children are much higher with the frequency of isolation from stools correlating with age. The carriage rate in neonates ranges from 52 – 64% (Enad *et al.,* 1997; Holst *et al.,* 1981; Larson *et al.,* 1982), most individuals being asymptomatic carriers. In infants under two years of age the carriage rate varies between 4% and 60% with a greater number of individuals being symptomatic (Holst *et al.,* 1981; McFarland *et al.,* 2000). The composition of the intestinal microbiota in early infancy is influenced by a number of factors. It has been shown that infants born by caesarean section are more often colonised with *C. difficile* and have lower numbers of bifidobacteria and *Bacteroides* spp than vaginally delivered infants (Penders *et al.,* 2006). This study also demonstrated that exclusively bottle-fed infants are more often colonised with *C. difficile* than breastfed infants and that hospitalisation and prematurity are also associated with higher counts of *C. difficile.* The organism is usually acquired from the environment rather than the mother: Matsuki *et al.,* (2005) demonstrated that children attending either a day-nursery or kindergarten were colonised with strains identical to those found on the floors of these facilities. A prospective study in a neonatal ward by Delmée *et al.,* (1988) showed that most of the neonates were colonised after admission by environmental strains.

1.4 The disease

1.4.1 Incidence of ribotypes among clinical isolates

A prospective study of *C. difficile* infections in Europe (Barbut *et al.,* 2007b) identified 66 different ribotypes, with 12 ribotypes (001, 002, 012, 014, 017, 020, 027, 048, 077, 078, 126 and 168) accounting for 65.5% of isolates studied. The distribution of ribotypes varies greatly from country to country. The prevalence of the ribotype 027/North American pulsed-field 1/BI strain (the so-called "hypervirulent" strain) was 6.2%. As of 2008 this ribotype had been reported in 16 European countries (Kuijper *et al.,* 2008).

Ribotypes 106 and 001 are the most common in Scotland. In the three months to December 2008 these ribotypes comprised 34.8% and 23.2% respectively of the isolates typed by the Scottish Reference Laboratory. Ribotype 027 comprised 10.9%, with 078 comprising 2.7% (HPS *C. difficile* Working Group, 2009a).

1.4.2 Nosocomial incidence

A mandatory surveillance programme, under which episodes of CDI in patients aged 65 and older are recorded, was introduced in Scotland in September 2006; 6,631 cases were reported in the year to September 2008, an increase of 0.1% on the year to September 2007 (HPS *C. difficile* Working Group, 2009b).

In England mandatory reporting of cases in patients aged 65 and older has been in place since January 2004. There was a steady increase in cases in this age group

between 2000 and 2006, peaking at 55,635 in the latter year, with a small decrease (9%) seen in 2007 (Health Protection Agency, 2009). Mandatory surveillance for the 2-64 years age group was introduced in April 2007. In the year to March 2008, infections in this age group accounted for 18% of the reported total.

1.4.3 Risk factors for nosocomial disease

A systematic review by Bignardi (1998) of published data highlighted frequently identified risk factors for CDI including: severity of co-morbidity; increasing age; duration of hospital stay; the duration of courses of antibiotics; the use of multiple antibiotics; nasogastric intubation; and the use of gastric acid suppressants.

Antibiotics

Previous treatment with antibiotics is widely acknowledged as a key risk factor, principally due to disruption of the native gut flora and hence the protection this flora provides against enteric pathogens. In the absence of such disruption *C. difficile* is unable to colonise the gut. Borriello & Barclay (1986) demonstrated that faecal emulsions containing viable organisms taken from healthy adult volunteers inhibited the growth of *C. difficile*; emulsions taken from patients receiving antibiotics had a reduced ability to inhibit growth. Similarly, caecal emulsions taken from clindamycin-treated hamsters did not display the inhibitory capabilities of emulsions from untreated hamsters (Borriello & Barclay, 1986). Clindamycin (Tedesco *et al.,* 1974) and β-lactamase-susceptible β-lactams (Starr *et al.,* 2003; Tedesco, 1975) are most commonly associated with CDI. However, most antibiotics, particularly oral antibiotics, have the potential to induce disease (Bartlett, 1992). The use of

clindamycin has declined since the 1970s and from the late 1980s the use of cephalosporins, in particular third-generation drugs, has carried the highest risk of CDI (Baxter *et al.,* 2008; Gerding, 2004). Starr *et al.,* (2003) found that the use of third-generation cephalosporins increased the risk of an individual becoming culture-positive. This study also showed that an individual's risk of becoming toxin-positive increased with the use of antibiotics, particularly amoxicillin and cephalosporins other than ceftriaxone. More recently, attention has turned to a possible role for fluoroquinolones in the aetiology of CDI. In a recent review Deshpande *et al.,* (2008) concluded that the data are suggestive of an association between fluoroquinolone use and CDI, in particular that caused by the 027 ribotype, but are not yet conclusive. Gerding (2004) suggests that frequent fluoroquinolone use in hospitals could be increasingly responsible for outbreaks of CDI for two reasons. Firstly, the newer fluoroquinolones have greater activity against anaerobes and are therefore more likely to disrupt normal flora. Secondly, they may exert selective pressure favouring the emergence of fluoroquinolone-resistant strains during antibiotic treatment and the subsequent proliferation of resistant clones.

Age

Nosocomial CDI is principally a disease of older individuals (65 years and above) (Al-Eidan *et al,* 2000; Asha *et al.,* 2006; Karlström *et al,* 1998; Kyne *et al.,* 1999; McFarland *et al.,* 1995; McFarland *et al.,* 1990; Starr *et al.,* 2003). This susceptibility likely stems from the increased probability in this population of serious co-morbidity, greater exposure to antibiotics and longer periods spent in hospitals, all identified risk factors (Bignardi, 1998). Colonisation resistance provided by gut

commensal flora is also reduced in the elderly (Borriello & Barclay, 1986; Hopkins & McFarlane, 2002).

Clinical procedures and treatments

Administration of antineoplastic drugs (Halim *et al.,* 1997) and diuretics (Raveh *et al.,* 2006) has been associated with CDI. Clinical procedures associated with increased risk of CDI include: the placement of nasogastric tubes (Asha *et al.,* 2006; Bliss *et al.,* 1998; Kyne *et al.,* 1999); administration of enemas (McFarland *et al.,* 1990); and endoscopy and gastrointestinal surgery (Kyne *et al.,* 1999; McFarland, 1998; Spencer, 1998).

Gastric acid suppressants

Suppression of the production of gastric acid by proton pump inhibitors (PPIs) and H_2 blockers, resulting in the loss of an innate defence mechanism against ingested pathogens, has been recognised as a risk factor for CDI by certain studies (Aseeri *et al.,* 2008; Choudhry *et al.,* 2008; Cunningham *et al.,* 2003; Dial *et al.,* 2004, 2005). However, Wilcox *et al.,* (2008) found that prior exposure to PPIs or H_2 antagonists was not significantly more common in CDI cases. The study by Choudhry *et al.,* (2008) reported that valid indications for the prescribing of PPIs could not be identified in 63% of the patients; it has been suggested that reducing the unnecessary use of PPIs may help reduce incidence of CDI.

A recent meta-analysis by Garey *et al.,* (2008) highlighted risk factors for recurrent CDI. They found that continued use of non- *C. difficile* treatment antibiotics after a

diagnosis of CDI, concomitant administration of gastric acid suppressants, and older age were significantly associated with increased risk of recurrent CDI.

Co-morbidity

A number of studies have demonstrated an association between susceptibility to CDI and underlying morbidity. Harbarth *et al.,* (2001) showed an association with the presence of gastrointestinal disease, anaemia and renal disease. CDI has also been reported in HIV-positive patients (Tumbarello *et al.,* 1995*)* and individuals with leukaemia (Milligan & Kelly, 1979). Patients with Crohn's disease and ulcerative colitis (collectively known as inflammatory bowel disease) have developed CDI in the absence of antibiotic exposure albeit CDI is more common when antibiotics have been administered (Greenfield *et al.,* 1983).

Wilcox & Fawley (2007) reported a correlation between CDI and viral gastroenteritis, with rates for CDI found to be higher in units affected by norovirus outbreaks. The reason for this association is unclear: it could result from an increase in the risk of transmission of *C. difficile* or it is possible that norovirus infection perturbs the commensal flora allowing colonisation by *C. difficile.*

1.4.4 Community-acquired CDI and the emergence of disease in previously low risk groups

CDI is classically regarded as a nosocomial infection. However, there is growing evidence of the importance of the community as a source of infection. Community-acquired CDI is defined by The European Centre for Disease Prevention and Control

as the onset of symptoms outside a health care facility with the patient not having been discharged from a health care facility within 12 weeks of symptom onset; or the onset of symptoms within 48 hours of admission to a health care facility with the patient having no prior stay in a health care facility in the 12 weeks preceding symptom onset (Kuijper & van Dissel, 2008). It is of note that community-acquired CDI is being reported in populations not previously thought to be at risk and in many cases there is no link with known risk factors.

In a retrospective study in North Carolina Kutty *et al.,* (2008) found that 34% (208/604) of patients with community-onset CDI met this definition of community-acquired disease. In Connecticut, a 2006 surveillance study reported an annual incidence of 6.9 cases of community-acquired CDI per 100,000 population (CDC, 2008). Of 241 patients, 59 (25%) had no underlying conditions and no exposure to inpatient health care in the preceding 12 months. Compared with the other patients this group was younger, 63% being aged 45 or younger, and in 21 (36%) cases there was no history of antimicrobial use in the previous three months. Wilcox *et al.,* (2008) undertook a prospective case-control surveillance study of community-derived faecal samples and reported that 2.1% of randomly selected faecal samples were positive for *C. difficile* cytotoxin. Of the 42 case patients detected 26 (62%) were aged 65 or younger. Exposure to antibiotics in the four weeks prior to testing was significantly more frequent among cases than controls and hospitalisation in the previous six months was seen to be significantly associated with CDI. However, there was no history of antibiotic use in the month before toxin detection in almost half the cases and approximately one-third had not been exposed to antibiotics or been recently hospitalised. Similar findings were reported by Dial *et al.,* (2008) in

their case-control study of patients aged 65 years or older admitted to hospital with community-acquired CDI. Approximately half of the cases had no exposure to antibiotics in the three months preceding admission.

Reports are now emerging of CDI cases among peripartum women, historically considered a low risk group. Ten such cases were identified in four US states in the period 2003-2005 (CDC, 2005). Of these 10 patients, 40% required hospitalisation and one died. Rouphael *et al.,* (2008) identified 10 cases in a one year period from 2005-2006. Nine patients had a history of antibiotic use in the three months prior to diagnosis and three had been hospitalised. Six patients were admitted to intensive care and death occurred in three instances.

The significance of *C. difficile* among the paediatric population has been the subject of debate for many years given that asymptomatic carriage among infants is not uncommon. Recent data, however, suggest that cases of CDI among children are increasing. A study by Benson *et al.,* (2007) in a children's hospital in Washington D.C. reported that the number of cases of community-acquired CDI presenting in the emergency room increased from 1.18 cases per 1,000 visits in 2001 to 2.47 cases per 1,000 visits in 2006. Sixty one percent of children with a toxin-positive stool sample were aged 2 years or older. Kim *et al.,* (2008) examined the annual incidence of CDI between 2001 and 2006 among in-patient children in 22 American children's hospitals. Over the study period, it increased from 2.6 to 4.0 cases per 1,000 admissions, with the median age of affected children being 4 years. Data for 2006 for the Lothian University Hospitals Trust showed 10.4 episodes of CDI per 10,000

inpatient days in the 0-18 age group, with 6.0 episodes of CDI per 10,000 inpatient days in the 0-13 age group (Reddy, personal communication).

1.4.5 Costs of CDI

CDI is responsible for a substantial economic burden. The acquisition of CDI in hospital usually results in an increased length of stay and a concomitant increase in direct hospital costs. In a study of an American tertiary care hospital, Song *et al.,* (2008) reported an additional stay of five and a half days carrying a cost of $6,326 (£4,258) per case. In a similar study in Germany, Vonberg *et al.,* (2008b) reported an additional stay of seven days incurring an additional cost of €7,147 (£6,099).

1.4.6 Outcomes of infection

Following colonisation with toxigenic *C. difficile* individuals may become asymptomatic carriers or develop disease. The carriage rate among healthy adults is typically 3 - 7% (Kato *et al.,* 2001; Kelly & LaMont, 1998) but is higher among hospitalised individuals and those in long-term care facilities. McFarland *et al.,* (1989) reported that 21% of patients whose stools were culture-negative on admission acquired *C. difficile* during their hospital stay and of these individuals 63% remained asymptomatic, giving a carriage rate among this group of approximately 13%. Riggs *et al.,* (2007) reported that the carriage rate among asymptomatic individuals during an outbreak in a care facility was 51%. The spectrum of clinical features seen in those who develop disease ranges from mild diarrhoea to PMC. Patients with severe disease commonly present with abdominal pain, profuse diarrhoea (frequently non-bloody), leucocytosis, nausea, fever,

anorexia and malaise (Monaghan *et al.,* 2008). On occasion patients may present with toxic megacolon or perforation, further severe complications of CDI, with no history of diarrhoea (Bartlett, 1992). Recurrent disease occurs in between 15% and 35% of patients with CDI, reflecting either the persistence of the original strain or re-infection by a new strain (Fekety *et al.,* 1997; Maroo & Lamont, 2006; Walters *et al.,* 1983).

1.5 Diagnosis

1.5.1 Clinical diagnosis

The presentation of an individual with diarrhoea and other characteristic symptoms after treatment with antibiotics, together with the characteristic nature of the stools can be used to diagnose CDI accurately (Wilcox, 2007). The use of flexible sigmoidoscopy (with biopsies) has been proposed for those patients suspected to have CDI but who have toxin-negative stools (Johal *et al.,* 2004a). PMC can be quickly diagnosed by endoscopic examination.

1.5.2 Laboratory diagnosis

This most commonly takes the form of toxin detection in stools. Many laboratories use commercially produced enzyme-linked immunosorbent assays (ELISAs) to detect toxin A or toxins A and B. It is preferable to use a test that detects both toxins given the existence of A^-B^+ strains that cause clinical symptoms. Stool samples may also be cultured on selective media such as CCEY which contains cycloserine, cefoxitin, egg yolk, lysed blood and sodium cholate to encourage the germination of

spores (Brazier, 1993). The organism is identified by colony morphology, odour, and fluorescence under UV light (Brazier, 1993). Due to the three to four day delay in obtaining a diagnosis, culture is not routinely carried out in hospital laboratories. It is, however, extremely valuable in investigating outbreaks as isolates can be characterised. A recently introduced assay for the detection of the glutamate dehydrogenase antigen (C.Diff Chek-60; TechLab) has proved to have a very high negative predictive value. However, due to its modest positive predictive value it is necessary to couple its use with a further confirmatory test such as the tissue culture cytotoxicity neutralisation assay. This increases the cost and time involved in testing and its use is unlikely to be widely accepted until a more cost-effective, rapid second test is introduced (Gilligan, 2008). A commercial real-time PCR assay (BD GeneOhm Cdiff) amplifying *tcdB* has been available since 2007 and its first evaluations have been recently published (Barbut *et al.,* 2009; Stamper *et al.,* 2009). The assay was found to be a more rapid (less than 3 hours) and more sensitive method than the tissue culture cytotoxicity neutralisation assay.

1.6 Treatment

1.6.1 Initial episode

Cessation of precipitating antibiotic treatment

In those with a mild case of CDI, stopping the precipitating antibiotic may be enough to resolve the problem. In a prospective randomised trial of metronidazole against vancomycin, diarrhoea resolved in 22.8% of patients in the 48-72 hour period before they were recruited into the study (Teasley *et al.,* 1983). In a more recent prospective

study (Johal *et al.,* 2004a) 30% of patients saw their diarrhoea resolve in a median period of 4 days post-cessation of precipitating antibiotic.

Antimicrobial treatment

In controlled clinical trials vancomycin, metronidazole, bacitracin, fusidic acid and teicoplanin have been shown to be effective in the treatment of CDI (Nelson, 2007). The most commonly prescribed treatments are oral metronidazole and oral vancomycin (Gerding *et al.,* 2008b). Until recently studies suggested there was little to choose between them in terms of patient response or recurrence rates (Teasley *et al.,* 1983; Wenisch *et al.,* 1996), although vancomycin has a quicker response time (Wilcox & Howe, 1995). It has now been reported (Zar *et al.,* 2007) that vancomycin is superior for the treatment of severe CDI. Reduced susceptibility to metronidazole among clinical isolates has been reported (Baines *et al.,* 2008) but the implications for clinical practice are not yet clear.

1.6.2 Relapses and recurrences

The risk of recurrent disease is higher in individuals who have had more than one episode of CDI (Kelly *et al.,* 1994) and it poses a therapeutic challenge. The use of tapered and pulsed regimens of vancomycin has been demonstrated to be beneficial (McFarland *et al.,* 2002). In an uncontrolled study by Johnson *et al.,* (2007) rifaximin given immediately after vancomycin treatment prevented predictable recurrences in seven of eight patients.

1.6.3 Role of surgery

Indications for surgery include toxic megacolon, perforation and failure to respond to medical therapies. Post-operative mortality rates are often high (Longo *et al.,* 2004; Synnott *et al.,* 1998).

1.6.4 Alternative therapies

Treatment alternatives to metronidazole and vancomycin are being keenly sought.

New antimicrobials

Agents under investigation include nitazoxanide, rifaximin and ramoplanin (Monaghan *et al.,* 2008). OPT-80, a macrocyclic antibiotic, was recently reported to resolve mild to moderate diarrhoea in 91% of cases in a phase 2 clinical trial (Louie *et al.,* 2009). A one month recurrence rate of 5% was noted.

Probiotics

Treating CDI through the replacement of disrupted intestinal flora by administration of probiotic organisms is intrinsically attractive. However, a recent systematic Cochrane review of published data (Pillai & Nelson, 2008) found that only one study reported a statistically significant benefit in the treatment of CDI. McFarland *et al.,* (1994) found that patients receiving *Saccharomyces boulardii* were significantly less likely to experience recurrences than patients receiving placebo. Pillai & Nelson concluded that there is insufficient evidence to recommend probiotic therapy as an adjunct to antibiotic therapy for CDI and that there is no evidence to support the use of probiotics alone in its treatment.

Immunotherapy

Case reports have indicated that the use of intravenous human immunoglobulin (IVIG) in patients with severe CDI is beneficial (McPherson *et al.,* 2006; Salcedo *et al.,* 1997). However, a small pair-matched study by Juang *et al.,* (2007) revealed no difference in clinical outcome between those treated with IVIG and those not.

Non-toxigenic strains

Sambol *et al.,* (2002) demonstrated that colonisation of the gut with non-toxigenic *C. difficile* (NTCD) post-administration of clindamycin protected hamsters after challenge with a toxigenic strain. The use of NTCD to prevent recurrent disease in humans will shortly be clinically trialled (ViroPharma Inc.). The aim of this therapy is to colonise the gut of patients treated for initial episodes of CDI with NTCD to prevent re-infection by toxigenic strains.

Toxin binding compounds

Treatment with these compounds is based on the assumption that preventing interaction between toxins and host cells will mitigate damage. Colestipol and cholestyramine, both anion-exchange resins, bind *C. difficile* toxins but no clinical efficacy has been demonstrated (Aslam *et al.,* 2005).

Tolevamer, the salt of an anionic polymer, binds both toxins A and B. Phase II clinical trials demonstrated non-inferiority to vancomycin for treatment of mild to moderate CDI (Louie *et al.,* 2006). However a study demonstrated that Tolevamer

failed to neutralise cytotoxicity in a human gut model, reflecting the poor efficacy seen in recent phase III trials (Baines *et al*., 2009).

Vaccines

The use of a vaccine containing toxoids A and B has been reported in a study of three patients with recurrent CDI (Sougioultzis *et al.,* 2005). In the six months following vaccination no further episodes of CDI were reported. Sanofi Pasteur is presently taking its A and B-toxoid-based candidate vaccine into phase II clinical trials (Sanofi Pasteur, 2009).

Faecal transplants

The use of healthy donor stools, administered as an enema or via nasogastric tube, to replace a patient's microbiota has met with some success. Aas *et al.,* (2003) reported in a retrospective review that 15 of 18 patients with recurrent CDI remained relapse free 90 days post-"transplant".

1.7 Control and prevention of CDI in health care facilities

Two interventions have been shown to be effective at reducing transmission during outbreaks of CDI: disinfection with 10% hypochlorite solution to reduce environmental contamination and use of barrier precautions (particularly gloves) by health care workers (Gerding *et al.,* 2008a). It is important that all surfaces commonly contaminated by spores are cleaned appropriately. Isolation or cohorting of patients with CDI and good hand hygiene practices by health care workers and

visitors in contact with patients is recommended. Exposure to antibiotics is a very important predisposing factor in CDI and their prudent use should be promoted as standard in attempts to prevent and control CDI (Vonberg *et al.,* 2008a). A recent Cochrane analysis (Davey *et al.,* 2005) indicated that hospital-acquired infections, in particular CDI, can be reduced by improvements in antibiotic prescribing. Effective antimicrobial stewardship programmes restrict the use of high-risk antibiotics, reduce polypharmacy and long-term therapy and advocate avoidance of inappropriate prescribing (Vonberg *et al.,* 2008a).

1.8 Virulence factors

1.8.1 The pathogenicity locus (PaLoc)

The PaLoc is a 19.6kb region of the chromosome, present in only one copy and at the same site in all strains of *C. difficile* (Braun *et al.,* 1996). It encodes five genes: toxin A (*tcdA*); toxin B (*tcdB*); *tcdC; tcdR* and *tcdE* (Figure 1.1). A pathogenic strain is defined as one that produces at least one of the two toxins. In non-pathogenic strains the PaLoc is absent (Cohen *et al.,* 2000b). Sequences at either side of the PaLoc are conserved in all strains suggesting that this region may be a distinct genetic element, albeit one that is not mobile (Braun *et al.,* 1996).

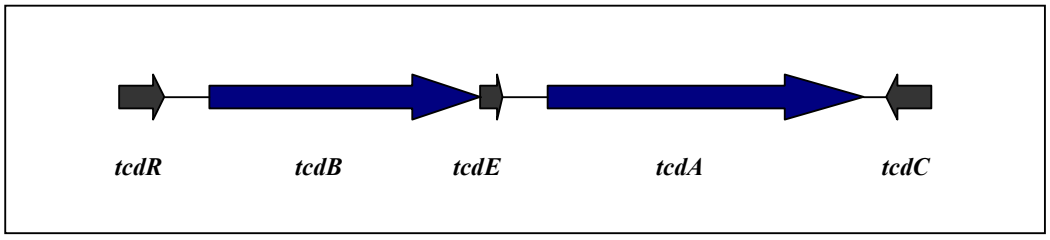


Figure 1.1. Schematic representation of the PaLoc (adapted from Matamouros *et al.,* 2007).

As discussed in section 1.2.2 the method of toxinotyping can be used to differentiate between strains. Strains which carry a PaLoc identical to that of reference strain VPI10463 are known as toxinotype 0; currently 24 variant toxinotypes (I – XXIV) are known (Rupnik, 2008). In addition to these toxin-gene-variants toxin-production-variants have been identified, characterised by the production of toxin B only or by the production of the binary toxin CDT.

A^-B^+ strains were the first well-characterised variant strains (Borriello *et al.,* 1992; Depitre *et al.,* 1993; Lyerly *et al.,* 1992). The A^-B^+ phenotype is currently associated with four toxinotypes: VIII, X, XVI and XVII, with the majority of strains being of toxinotype VIII (Rupnik, 2008). In this toxinotype the lack of TcdA production stems from the presence of a nonsense mutation introducing a stop codon in *tcdA* (von Eichel-Streiber *et al.,* 1999). Until recently it was thought that both toxins were required to cause disease, however outbreaks of CDI caused by A^-B^+ strains are being reported more frequently, undermining this belief (al-Barrak *et al.,* 1999; Johnson *et al.,* 2001; Kuijper *et al.,* 2001; Limaye *et al.,* 2000).

1.8.2 The mechanism of action of toxins A and B

Toxins A and B, considered the main virulence factors of *C. difficile*, are part of a group of toxins referred to as "Large Clostridial Toxins" (LCTs). Toxins A and B are exotoxins which are taken up from the gut lumen by enterocytes. Initial investigation of the toxins suggested that only toxin A was enterotoxic. Both are cytotoxic in cell cultures although toxin B is 100 to 1000 times more potent in this regard than toxin A; toxin B is therefore referred to as the cytotoxin and toxin A as the enterotoxin,

inducing diarrhoea (Schirmer & Aktories, 2004). Data from animal work (Corthier *et al.,* 1991; Kurtz *et al.,* 2001) led to the proposal that toxin A is the major pathogenic toxin in CDI, however, a recent study by Lyras *et al.,* (2009) provides evidence that toxin B is the more important of the two. This group produced *tcdA* and *tcdB* mutants in which the separate toxin genes were inactivated and the virulence of the mutants tested in the hamster model of disease. It was found that *tcdA* mutants remained fully virulent, while the *tcdB* mutants had an attenuated virulence phenotype.

Toxins A and B have very similar molecular weights, amino acid sequences and enzymatic activity; both glucosylate small GTPases (Voth & Ballard, 2005). They have three regions: the N-terminal catalytic domain; the C-terminal toxin receptor-binding domain; and a putative translocation domain (Figure 1.2).

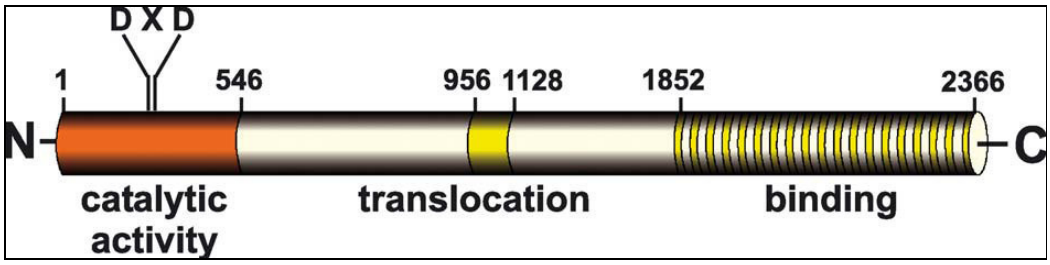


Figure 1.2. The structure of toxin B (taken from Schirmer & Aktories, 2004).

Binding to the toxin receptor

The C-terminal binding domain is composed of repetitive oligopeptide elements, known as CROPs, which are regarded as host cell surface polysaccharide-binding motifs (Just & Gerhard, 2004). The receptors for the LCTs are believed to be nonproteinaceous. TcdA has been shown to bind to a disaccharide present on the I, X

and Y antigens found on a variety of human cells (Tucker & Wilkins, 1991). TcdB has been shown to bind to a number of different cell types although its receptor remains unidentified (Voth & Ballard, 2005). Toxin-receptor interaction induces receptor-mediated endocytosis (Just & Gerhard, 2004).

Membrane translocation

Translocation of the toxins into the cytosol requires acidification of the early endosome. Barth *et al.,* (2001) demonstrated that inhibition of toxin B cytotoxicity resulted from treatment of cells with a proton pump inhibitor. Furthermore, if the medium was acidified the toxin could be taken up directly into the cytosol in spite of the presence of the inhibitor. In response to the lowered pH in the endosome the toxins undergo a conformational change, exposing hydrophobic domains (Qa'Dan *et al.,* 2000) which facilitate interaction with the endosomal membrane (Barth *et al.,* 2001). The question of whether the full toxin B or the enzymatic domain alone is translocated into the cytosol was recently answered by Pfeifer *et al.,* (2003). A number of techniques were used to demonstrate that the C-terminal remained in the endosome while the N-terminal was located in the cytosol. The toxin is autocatalytically cleaved by a cysteine protease activated by inositol hexakisphosphate (Egerer *et al.,* 2009).

Enzymatic activity

The cytosolic targets of TcdA and TcdB are the proteins of the Rho and Ras families (Just *et al.,* 1995a, 1995b). These proteins belong to a superfamily of low molecular mass GTPases which act as molecular switches in a large number of signalling

pathways. The GTPases alternate between an inactive, GDP-bound form in the cytosol and an active, GTP-bound form at the cell membrane. In the cytosol the Rho proteins bind the guanine nucleotide dissociation inhibitor (GDI), this appears to prevent nucleotide exchange. The GTPases translocate to the cell membrane following receptor-mediated activation; here GDP is exchanged for GTP in a process catalysed by guanine exchange factors (GEFs). GTP is subsequently hydrolysed to GDP by members of the GTPase activating proteins family (GAPs) returning the GTPase to its inactive form (Schirmer & Aktories, 2004). Both TcdA and TcdB glucosylate the target protein by transferring a sugar moiety to threonine-37 of the GTPase using the co-substrate UDP-glucose (Just *et al.,* 1995a, 1995b), resulting in the GTPase's inactivation. Cycling of the Rho proteins under normal circumstances and the points of action of TcdA and TcdB are shown in Figure 1.3.

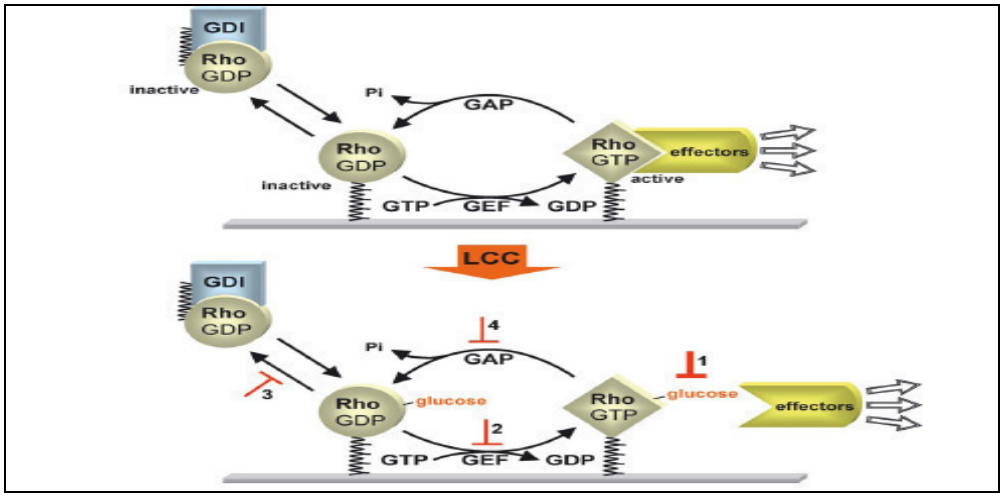


Figure 1.3. Glucosylation of GTPases by TcdA and TcdB (taken from Schirmer & Aktories, 2004). Glucosylation by toxins A and B (LCC) blocks the coupling of Rho-GTPases with effector molecules (1); inhibits GEF-catalysed nucleotide exchange (2); blocks the Rho/GDI interaction (3); inhibits GTP hydrolysis activated by GAPs (4).

The glucosyltransferase activity is thought to result from the presence of a DXD (aspartate-X-aspartate) motif in the enzymatic domain (Busch *et al.,* 1998).

Effects of glucosylation on the functioning of GTPases

Rho GTPases regulate a number of different processes within the cell, hence their glucosylation results in a range of effects. Lethal depolymerisation of the actin cytoskeleton resulting in the typical rounding phenotype known as CPE (cytopathic effect) is prominent amongst these. Inactivation of the GTPases also results in the breakdown of maintenance of tight junctions within the colonic epithelial barrier. This increases the permeability of the epithelial layer, resulting in diarrhoea (Voth & Ballard, 2005). Neutrophils pass through the opened junctions and their presence in the lumen, together with mucus, fibrin and inflammatory cells released by inflammatory ulcers, contributes to the formation of a pseudomembrane (Thelestam & Chaves-Olarte, 2000). Cell apoptosis is an additional consequence of the glucosylation of GTPases (Calderon *et al.,* 1998; Zhao *et al.,* 2003).

The process of cellular intoxication, glucosylation and its effects on the Rho proteins is summarised in Figure 1.4.

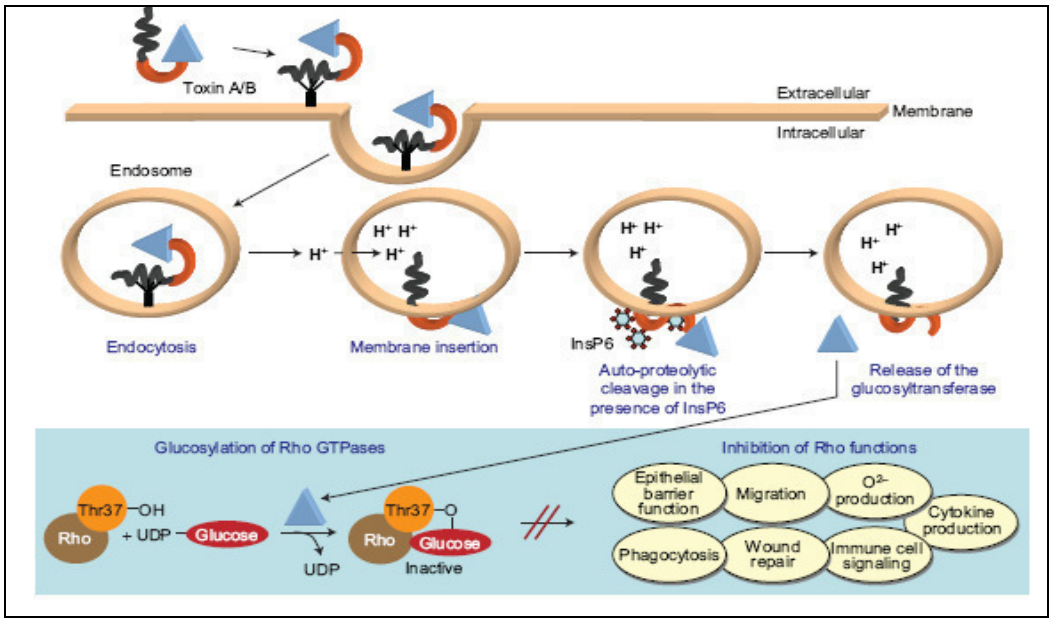


Figure 1.4. Representation of toxin entry, glucosylation and its effects on the Rho proteins (taken from Aktories, 2007).

1.8.3 TcdR

TcdR was proposed to be a positive regulator of the production of toxins A and B by Hundsberger *et al.,* (1997). Mani & Dupuy (2001) showed that TcdR acted as an alternative sigma factor that bound to the RNA polymerase core enzyme and activated transcription of *tcdA* and *tcdB* by mediating the binding of the enzyme to the toxin genes' promoters.

1.8.4 TcdC

Analysis of the transcription of the PaLoc (Hundsberger *et al.,* 1997) demonstrated that *tcdC* is transcribed divergently from the remainder of the genes and is highly expressed during exponential phase, with transcription shut off as the cells enter stationary phase. This pattern is the reverse of that seen in transcription of the toxin

genes and *tcdR,* suggesting that TcdC is a negative regulator of toxin gene expression. Evidence supporting this suggestion has been provided by Matamouros *et al.,* (2007) and Dupuy *et al.,* (2008). It was shown that TcdC destabilises the RNA polymerase-TcdR complex, preventing it from recognising and binding the *tcdA* and *tcdB* promoters and thus inhibiting toxin gene expression.

1.8.5 TcdE

The *tcdE* gene is co-transcribed with *tcdA* and *tcdB*; it encodes a hydrophobic protein homologous to the cytolytic holin proteins of bacteriophages (Tan *et al.,* 2001). This group demonstrated that TcdE acts at the cell membrane and that the expression of *tcdE* in *Escherichia coli* resulted in lysis of the bacterial cells unrelated to environmental factors. These findings prompted the suggestion that TcdE functions in the formation of membrane lesions that permit the release of toxins A and B into the environment. It is known that TcdA and TcdB lack a signal sequence mediating trafficking to the cell membrane and are thus likely to accumulate in the cytoplasm (Aktories, 1997). Why secretion of toxins is associated with bacterial cell death is unknown although it has been shown that toxin release correlates with sporulation and the survival of the organism in this form (Kamiya *et al.,* 1992).

1.8.6 Expression of the PaLoc

Hammond *et al.,* (1997) studied expression of the PaLoc by hybridisation of gene-specific probes with RNA transcripts. The data suggested a model in which the PaLoc is transcribed as a 17.5kb polycistronic transcript comprising *tcdA, tcdB, tcdR* and *tcdE*. It was shown that the transcript of *tcdA* was immediately processed while

that of *tcdB* formed part of a second processing intermediate along with the transcripts of *tcdR* and *tcdE.* Furthermore it was posited that *tcdA* and *tcdB* were also transcribed monocistronically. Further evidence of this mode of transcription was provided by Hundsberger *et al.,* (1997). They demonstrated that the PaLoc is organised with several promoters that control the simultaneous transcription of monocistronic transcripts of *tcdA* and *tcdB* and polycistronic transcripts of all four genes.

Regulation of transcription is multi-factorial. CodY, a global regulator of gene expression in low G+C Gram-positive bacteria, has been shown to repress toxin gene expression in *C. difficile.* Dineen *et al.,* (2007) demonstrated that inactivating the *codY* gene resulted in the derepression of the five genes of the PaLoc during exponential and stationary phases. CodY was found to bind to the *tcdR* promoter region with high affinity suggesting that regulation of toxin gene expression by this protein is mediated by direct control of *tcdR* expression. CodY was also found to bind with high affinity to the likely promoter region of *tcdC.* It is not known why CodY would repress both the positive and negative regulators of toxin gene expression. Binding to the *tcdR* promoter was enhanced in the presence of certain amino acids and GTP indicating a possible link between nutrient limitation and PaLoc expression.

The possibility of regulation by quorum sensing has been raised by the finding within the *C. difficile* strain 630 genome of the $luxS_{Cd}$ gene which encodes an AI-2 synthase and is primarily expressed in late exponential phase (Carter *et al.,* 2005). This gene is conserved in pathogenic and non-pathogenic strains (Lee & Song, 2005) and is

preceded by two genes known as *rolA* and *rolB* encoding proteins with similarities to bacterial two-component signalling systems (Carter *et al.,* 2005). The involvement of this system in transcriptional regulation of the PaLoc has yet to be definitively demonstrated.

1.8.7 Environmental regulation of the PaLoc

Toxin production is well known to be regulated by environmental factors. It is highly dependent on temperature (Karlsson *et al.,* 2003); maximal yields are seen at 37°C with low production at 22°C and 42°C. The presence in the growth medium of amino acids, in particular proline, cysteine and cysteine derivatives, down-regulates toxin production (Karlsson *et al.,* 1999, 2000). Thioglycollate does not impact upon toxin production, indicating that the effect of cysteine on toxin production is not mediated by changes in redox potential (Karlsson *et al.,* 2000). A coupling between toxin expression and up-regulation of enzymes involved in alternative energy metabolism, such as the use of butyrate or succinate, has been demonstrated (Karlsson *et al.,* 2000, 2008). It appears these pathways may be simultaneously altered by the presence of certain amino acids. Increasing concentrations of glucose in the growth medium reduces transcription of *tcdA* and *tcdB*, being completely repressed at 1% (Dupuy & Sonenshein, 1998). It has been hypothesised that glucose acts indirectly by preventing the exhaustion of certain amino acids, the presence of which represses toxin production (Karlsson et *al.,* 2008).

Reducing the concentration of biotin in the growth medium reduces growth and increases toxin production (Yamakawa *et al.,* 1996*)*. Conversely, an excess of biotin

inhibits the production of toxin while increasing growth (Yamakawa *et al.,* 1998). It has been suggested that biotin levels in the gut could be reduced as a result of the disruption of the commensal flora, triggering toxin production (Yamakawa *et al.,* 1996). The effects on growth and toxin production of adding amino acids to a growth medium in which biotin is limited differ between amino acids (Yamakawa *et al.,* 1998). The presence of lysine, asparagine, glutamine and glutamic acid repress toxin production. Growth is enhanced by the presence of asparagine, glutamine and glutamic acid and inhibited by lysine.

1.8.8 Antibiotics and expression of the PaLoc

The role of antibiotics in the pathogenesis of CDI through the impairment of colonisation resistance has already been discussed. A number of studies suggest that subinhibitory levels of antibiotics may further contribute to pathogenesis by influencing the production of toxins. Onderdonk *et al.,* (1979) showed that sub-MIC concentrations of penicillin and vancomycin increased toxin production, while Honda *et al.,* (1983) reported a toxin-inducing effect for cephalosporins and clindamycin. Drummond *et al.,* (2003b) demonstrated that sub-MIC concentrations, particularly of metronidazole, clindamycin and amoxicillin, induced toxin production earlier than was seen in antibiotic-free medium. Similar findings were reported by Gerber *et al.,* (2008). Strains exposed to sub-MIC levels of metronidazole, vancomycin, clindamycin and linezolid showed earlier toxin production. The transcription rate of toxin genes was increased by exposure to all antibiotics used apart from clindamycin. Similar findings in terms of effect on toxin production were reported by Sánchez-Hurtado (2007). However, this study reported different data in respect of PaLoc gene transcription. In the presence of amoxicillin *tcdA, tcdR* and

tcdE expression was up-regulated while that of *tcdB* was unaffected. Vancomycin showed no effects on transcription while clindamycin increased expression of all PaLoc genes. Metronidazole was shown to down-regulate *tcdC*. It should be noted that these data, unlike those of Gerber *et al.*, refer to a single time point and that changes of expression may have occurred at time points other than that analysed.

It is not possible to extrapolate from these *in vitro* findings to the situation *in vivo*, however, the use of a three-stage human gut model has attempted to address this. Freeman *et al.,* (2007) showed that sub-MIC levels of metronidazole increase toxin titres. In a study examining the effects in the gut model of cefotaxime and its active metabolite desacetylcefotaxime it was shown that *C. difficile* remained in a steady state during non-antibiotic exposed periods, with no detectable cytotoxin. However, exposure to both antibiotics induced proliferation of *C. difficile* and elevated cytotoxin levels (Freeman *et al.,* 2003). Conversely, the use of tigecycline was shown not to induce proliferation or cytotoxin production (Baines *et al.,* 2006).

1.8.9 Other virulence factors

Binary toxin

A strain of *C. difficile* that produced a third toxin, known as the binary toxin, was isolated by Popoff *et al.,* in 1988. This binary toxin (CDT), an actin-specific ADP-ribosyltransferase, has similar characteristics to the binary toxins of other clostridia and is unrelated to TcdA and TcdB. It does not produce the cytopathic effect typically induced by TcdA and TcdB. CDT consists of two subunits: CDTa and CDTb. CDTa is the enzymatic subunit that ADP-ribosylates monomeric actin,

resulting in cytoskeletal disruption. CDTb is the binding subunit responsible for the translocation of the enzymatic subunit into the cytoplasm (Barth *et al.,* 2004).

The prevalence of the binary toxin genes is relatively low. A European prospective study reported that 17.2% of toxigenic strains carried the binary toxin genes; all these strains were of variant toxinotypes (Barbut *et al.,* 2007b). The role of binary toxin in the pathogenesis of CDI is not understood. While it is clearly not key to the development of disease, it has been suggested that it contributes to its severity. Barbut *et al.,* (2007a) reported that binary toxin-positive strains, of varying ribotypes, were associated with more severe diarrhoea and with a higher case-fatality rate. Analysis of a collection of clinical samples by Geric *et al.,* (2003) identified A^-B^- strains carrying the binary toxin genes that had been isolated from patients with diarrhoea. They could not, however, determine whether the strains had been the cause of the diarrhoea. Binary toxin preparations have been shown to induce fluid accumulation in the rabbit ileal loop model (Geric *et al.,* 2006). However, challenge with A^-B^- CDT^+ strains in a hamster model produced colonisation but no evidence of disease (Geric *et al.,* 2006). It was posited that binary toxin alone does not cause disease but may act as an adjunct to TcdA and TcdB in pathogenesis.

Surface proteins

Borriello *et al.,* (1987) hypothesised that adherence to the gut mucosa was an important contributor to the virulence of *C. difficile*. A number of cell-surface proteins have been identified as adhesins.

The S-layer is a protective surface that contributes to the maintenance of envelope rigidity and shape of certain bacterial cells. In *C. difficile* the S-layer is comprised of

two subunits, derived from a common precursor encoded by the *slpA* gene (Calabi *et al.,* 2001). These S-layer proteins have been shown to adhere to gut epithelial cells (Calabi *et al.,* 2002).

Other surface proteins identified as putative adhesins include Cwp66, GroEL, Fbp68 and FliD (Calabi *et al.,* 2002; Cerquetti *et al.,* 2000; Hennequin *et al.,* 2001b, 2003; Karjalainen *et al.,* 2001; Waligora *et al.,* 2001). Cwp66 ("clostridial wall protein 66 KDa") is a surface heat-shock protein with a two domain structure (Waligora *et al.,* 2001). Its homology to known proteins indicates that its N-terminal domain is likely to be involved in adhesion. GroEL (Hsp60) is another heat-shock protein, the expression of which is up-regulated in response to stressors such as heat, lack of iron, acid, high osmolarity and antibiotics (Hennequin *et al.,* 2001a). Fbp68 is a fibronectin-binding protein, binding both soluble and immobilised fibronectin (Hennequin *et al.,* 2003). Tasteyre *et al.,* (2001) showed that FliD, the flagellar cap protein, adhered to mucus, an important first step in gut colonisation.

Exposure to sub-MIC concentrations of ampicillin and clindamycin have been shown to moderately up-regulate expression of Cwp66, Fbp68 and S-layer proteins (Denève *et al.,* 2008). Overexpression was correlated with increased adherence of *C. difficile* to cultured cells under the same conditions.

Sánchez-Hurtado & Poxton (2008) investigated a possible modulating effect of cell-surface antigens on the activity of *C. difficile* toxin A on Vero and Caco2 cells. They found that in the presence of an EDTA extract containing several major and minor cell-surface proteins and the membrane-associated lipocarbohydrate (LC) or a

preparation of free LC the cytotoxicity of suboptimal levels of toxin A was significantly enhanced in both cell types.

C. difficile has a polysaccharide capsule that may be involved in adhesion and could contribute to evasion of the host immune system through its anti-phagocytic properties (Davies & Borriello, 1990). Complement components and antibodies do not bind as efficiently to encapsulated organisms, impairing the phagocytes' ability to engulf the bacterial cell and protecting against complement-mediated lysis. In certain organisms the capsule mimics the composition of human tissue; for example the capsule of *Streptococcus pyogenes* is composed of hyaluronic acid, a polymer present in human connective tissue. The capsule thus prevents recognition of the organism by the host immune system.

1.9 Immune response

1.9.1 The immune system of the gut

The intestine encounters more microbial antigen than any other part of the body; in addition to the commensal flora of the gut many pathogenic bacteria, viruses and parasites enter the body through the intestinal mucosa. The immune system of the gut has evolved to maintain the number and composition of commensal organisms while ensuring they remain within the gut lumen. Furthermore it acts to neutralise pathogens and maintains the integrity of the gut epithelium by minimising inflammatory tissue damage (Holmgren & Czerkinsky, 2005). This immune protection is provided by the gut-associated lymphoid tissues (GALT). GALT comprise the Peyer's patches of the small intestine, the mesenteric lymph nodes and

the isolated lymphoid follicles of the large intestine. These tissues provide the site of induction of the adaptive immune response. The effector cells generated here are found throughout the epithelium and lamina propria of both the small and large intestine (Platt & Mowat, 2008). A key feature of the mucosal immune system is the production of large quantities of IgA. Dimers of IgA are secreted into the gut lumen through the interaction with the polymeric Ig receptor expressed on the basolateral surface of epithelial cells (Mostov & Deitcher, 1986). Secretory IgA (SIga) inhibits bacterial adhesion, neutralises viruses and bacterial toxins and helps eliminate antigen from tissue by transporting it back into the lumen and promoting its uptake by phagocytic cells (Holmgren & Czerkinsky, 2005). Importantly, it also promotes the maintenance of commensal bacterial populations (Fagarasan *et al.,* 2002).

The presence of microbes is detected by cells of the innate immune system, including dendritic cells (DCs), macrophages and monocytes, through Toll-like receptors (TLRs). Individual TLRs recognise distinct pathogen-associated molecular patterns (PAMPs) that have been evolutionarily conserved in specific classes of microbes. Binding of these PAMPs to TLRs initiates events that usually result in the increased expression of pro-inflammatory genes (Cook *et al.,* 2004). The anti-inflammatory milieu of the gut is maintained by a phenotypically distinct population of immune cells. Mucosal antigen-presenting DCs have been shown to be conditioned by epithelial cells to initiate polarised T_H2 helper cell responses, characterised by the secretion of anti-inflammatory cytokines including IL-10, IL-4 and TGF-β (Rimoldi *et al.,* 2005). These cytokines promote IgA class switching and production by activated B cells (Cerutti & Rescigno, 2008). DCs may also activate B cells to

produce IgA in a T cell-independent manner through the production of a B cell-activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL) (He *et al.,* 2007). Recognition of PAMPs by TLRs present on DCs and intestinal epithelial cells stimulates production of BAFF and APRIL by both cell types (He *et al.,* 2007). Intestinal macrophages differ from their peripheral counterparts in a number of ways. For instance, while they are highly phagocytic they fail to produce pro-inflammatory cytokines after the recognition of PAMPs by TLRs. It has been shown that this results from low expression of TLRs (Hausmann *et al.,* 2002). Thus a major role of the intestinal macrophages is to maintain homeostasis. Protection against pathogens through the induction of an inflammatory response appears to be provided by the recruitment of a population of monocytes expressing a full range of TLRs (Platt & Mowat, 2008).

1.9.2 The inflammatory response to CDI

Colitis in CDI is characterised by a massive influx of neutrophils into the colonic mucosa and the pseudomembranes of PMC contain abundant dead neutrophils. IL-1, IL-8, TNF-α and leucotriene B4, produced by mucosal immune and epithelial cells, recruit neutrophils from the circulation to the site of inflammation (Souza *et al.,* 1997). *In vitro,* cultured monocytes can be stimulated to release TNF-α by both toxin A and toxin B (Souza *et al.,* 1997). *In vitro* exposure to toxigenic *C. difficile* or to supernatants from toxigenic strains stimulates IL-8 production by intestinal epithelial cells (Canny *et al.,* 2006). Both toxins also activate monocytes and macrophages in the lamina propria *in vitro* to release IL-8 (Linevsky *et al.,* 1997).

1.9.3 Host immune response to *C. difficile*

It has been suggested that the differing clinical presentation of CDI reflects host rather than bacterial factors. A number of studies have addressed host immune response to *C. difficile*.

Humoral response in initial episodes of CDI

Kyne *et al.,* (2000) performed a prospective study of the humoral response of patients colonised by *C. difficile* while hospitalised. Of the 47 patients identified 19 remained asymptomatic. Kyne *et al.,* showed that asymptomatic carriage was associated with an effective anamnestic response to toxin A in which high levels of serum anti-toxin A IgG developed rapidly after colonisation. Conversely, those colonised patients with low levels of serum anti-toxin A IgG had a much greater risk of developing symptomatic disease. The levels of serum IgM against non-toxin antigens (products of sonication of non-toxigenic strains) were also found to be significantly higher in asymptomatic individuals. Levels of serum anti-toxin B IgG and anti-toxins IgM and IgA were not significantly different between the two groups. Furthermore, Kyne *et al.,* identified the presence of severe underlying disease on admission as a risk factor for symptomatic disease. Colonised patients with severe or extremely severe underlying disease were eight times more likely to develop diarrhoea than those with less severe underlying disease. All colonised patients with high serum anti-toxin A IgG and less severe underlying disease were asymptomatic carriers while those with low levels of antibody and severe underlying disease were symptomatic.

Péchiné *et al.,* (2005) compared serum antibody levels in cases and controls to the adhesins Cwp66-Nterminal, Cwp66-Cterminal, FliC, FliD, Fbp68 and toxins A and B. They found that controls had a significantly higher level of antibodies to Cwp66-Nterminal, FliC, FliD and Fbp68 than did cases: antibody levels to toxins A and B did not differ significantly. Drudy *et al.,* (2004) analysed serum antibody levels to surface layer proteins in a population of hospitalised patients divided into case, carrier and control groups. No significant differences were found in levels of anti-SLP IgG, IgM or IgA. A study by Sánchez-Hurtado *et al.,* (2008) compared the antibody response to colonisation in confirmed cases of CDI, asymptomatic carriers and non-colonised asymptomatic controls. Levels of IgG to antigens other than lipocarbohydrate were highest in cases and lowest in controls. Antibodies to lipocarbohydrate were higher in controls than cases. Levels of antibodies in carriers were found to be similar to those in controls or of an intermediate level. The levels of IgM to all antigens were not significantly different among all three groups. The authors suggest that the immunological basis of predisposition to symptomatic disease may lie in the timing or specificity of the humoral immune response mounted.

Humoral response in recurrent CDI

Kyne *et al.,* (2001) conducted a prospective study of a population with nosocomial CDI in which serum levels of IgG, IgA and IgM against toxins A and B and non-toxin antigens were measured over a sixty-day period. Of the 44 participants 22 had recurrent diarrhoea. On day three of the first episode of diarrhoea patients who experienced a single episode had higher serum levels of anti-toxin A IgM than those

who experienced multiple episodes. On day 12 patients who had a single episode had higher serum levels of anti-toxin A IgG than those who experienced recurrent diarrhoea. The authors also reported that patients with recurrent diarrhoea were more likely to be older, have more severe underlying disease and to receive more antibiotics than patients experiencing a single episode. The odds ratio for recurrence associated with a low level of serum anti-toxin A IgG at 12 days post-onset of the initial episode of diarrhoea was 48.0 (95% CI 3.5-663) and the authors concluded that a serum antibody response to toxin A during the initial episode was protective against recurrence. Katchar *et al.,* (2007) reported significantly lower levels of anti-toxin A IgG2 and IgG3 subclasses in individuals with recurrent CDI.

Drudy *et al.,* (2004) examined the association between serum anti-SLP antibodies and recurrent diarrhoea and found that those patients experiencing multiple episodes had significantly lower levels of anti-SLP IgM than those experiencing a single episode.

Mucosal cellular immune response

Johal *et al*., (2004b) studied immune cell populations in colonic biopsies from controls with diarrhoea not caused by *C. difficile* and from patients with either mild or severe CDI. They found that T cell counts did not differ between the groups. There were, however, significant reductions in B/plasma cells and macrophage counts in CDI patients compared with controls. Analysis of the antibody class produced by B/plasma cells showed that there were significant reductions in IgA-producing cells in biopsies from patients with CDI, with the greatest reduction being

seen in those from patients with PMC. On the other hand, IgG-producing cells were seen to be significantly increased in biopsies from patients with CDI. Analysis of patients who relapsed revealed that only those with PMC did so and that biopsies from these patients gave significantly lower B/plasma cell and IgA-producing cell counts than biopsies from those patients with PMC who did not relapse.

1.10 Fluoroquinolone resistance

Bacterial resistance to fluoroquinolones is mediated by different mechanisms including mutations of DNA gyrase (the subunits of which are encoded by *gyrA* and *gyrB)* and topoisomerase IV genes (*parC* and *parE*), reduced permeability and the presence of efflux pumps (Higgins *et al.,* 2003). Analysis of its genome has shown that *C. difficile,* in common with other organisms such as *Treponema pallidum* and *Helicobacter pylori,* lacks the genes for topoisomerase IV (Dridi *et al.,* 2002). It has been suggested that the presence of mutations in both *gyrA* and *parC* in *E. coli* leads to higher levels of fluoroquinolone resistance (Bachoual *et al.,* 2001), the implication being that lower levels of resistance may therefore be seen in *C. difficile*.

Fluoroquinolone resistant isolates of *C. difficile* characterised to date have shown amino acid changes in the quinolone resistance determining region (QRDR) of GyrA and GyrB. GyrB changes are less common than GyrA and tend to confer low levels of resistance (Spigaglia *et al.,* 2008). The GyrA amino acid substitution Thr82→ Ile has been reported by a number of groups (Ackermann *et al.,* 2001; Dridi *et al.,* 2002; Drudy *et al.,* 2007; Schmidt *et al.,* 2007; Spigaglia *et al.,* 2008). This substitution has been demonstrated in all ribotype 027 strains characterised so far (Drudy *et al.,* 2007;

Spigaglia *et al.,* 2008). The presence of this substitution was associated with a high level of resistance to all fluoroquinolones tested by Spigaglia *et al.,* (2008). Thr82 corresponds to Ser83 in *E. coli,* the substitution of which alters the structure of the quinolone binding site near the interface of the enzyme and DNA (Hooper, 1999). Less frequently detected GyrA substitutions include Asp71→Val, Ala118→Thr (Dridi *et al.,* 2002) and Thr82→Val (Ackermann *et al.,* 2001). Drudy *et al.,* (2006) reported the presence of the GyrB substitution Asp426→Val in a cluster of 5 clonal toxin-A-negative, toxin-B-positive outbreak isolates found to be resistant to five fluoroquinolones. Asp426 is thought to be a key site in GyrB; mutations in the corresponding codon in *E. coli, Staphylococcus aureus* and *Streptococcus pneumoniae* are associated with fluoroquinolone resistance (Hooper, 1999). No substitutions in GyrB have been noted to date in ribotype 027 strains (Drudy *et al.,* 2007; Spigaglia *et al.,* 2008).

Efflux pumps are known to be present in many bacteria. They can confer resistance to a variety of molecules, including antibiotics, by extrusion (Lebel *et al.,* 2004). Little is known as yet about the role of efflux pumps in multidrug resistance in *C. difficile.* Dridi *et al.,* (2004) cloned a gene designated *cdeA* (*C. difficile* efflux) under the control of its natural promoter into *E. coli* and *Clostridium perfringens.* It rendered the host cell resistant to ethidium bromide and acriflavin but not to a number of antibiotics including the fluoroquinolones norfloxacin and ciprofloxacin. However, when cloned and overexpressed under the control of the strong P*lac* promoter it conferred fluoroquinolone resistance on *E. coli.* BLAST analysis

revealed that the CdeA protein sequence was homologous to those of members of the MATE (multidrug and toxic compound extrusion) family of efflux pumps.

Lebel *et al.,* (2004) studied a second putative efflux gene designated *cme* (*Clostridium* multidrug efflux), homologous to members of the major facilitator superfamily such as NorA from *Staph. aureus*, Bmr from *Bacillus subtilis* and PmrA from *Strep. pneumoniae*. The *cme* gene conferred resistance to ethidium bromide, safranin O and erythromycin, but not norfloxacin, when cloned into *Enterococcus faecalis.* Susceptibility to these compounds was increased in the presence of reserpine, a selective inhibitor of proton motive force-driven efflux pumps.

Immediately upstream of the *cme* gene in the *C. difficile* genome is a gene designated CD3197, a putative MerR family transcriptional regulator. Members of this family have been shown to activate suboptimal sigma 70-dependent promoters in which the -35 and -10 elements are separated by more than the optimal 17+/-1 bp (Brown *et al.,* 2003). Transcription is activated through protein-mediated DNA distortion and the majority of MerR regulators respond to environmental stressors such as the presence of antibiotics.

1.11 Aims of the thesis

This thesis consists of three studies:

1. A comparative study of immune cell populations within the lamina propria of colonic tissue taken from individuals either attending a colorectal surgery

outpatients' clinic or undergoing elective colorectal surgery at the Western General, a large Acute hospital in Edinburgh. The study participants were divided into three groups on the basis of *C. difficile* colonisation status: cases (symptomatically colonised); carriers (asymptomatically colonised) and controls (asymptomatic and not colonised).

The aim of this study was to quantify and compare effector T cells, B cells, plasma cells and macrophages within colonic tissue not exhibiting pathological changes taken from cases, carriers and controls. Previous research (Johal *et al.,* 2004) reported significantly decreased numbers of IgA-secreting B/plasma cells and macrophages in cases compared with controls. Cases also showed significantly greater numbers of IgG-secreting cells. The study performed by Johal *et al.,* reported findings from 12 controls and 16 cases, this population being of sufficient size to detect statistically significant differences. In the present study findings from six controls, six carriers and seven cases are reported. While this study is therefore underpowered the findings may still prove of use if incorporated into a metanalysis. The hypothesis for the present study was that comparable immune cell counts would be seen in carriers and controls, with both being higher than those seen in cases.

2. A number of studies have highlighted an association between the presence of certain host polymorphisms in genes associated with immune response and susceptibility to a variety of infections and diseases. This study focused on polymorphisms identified in the *TLR2, TLR5* and *IL-8* genes.

TLR2 has been shown to be important in host defence against Gram-positive organisms. A polymorphism in the human *TLR2* gene (Arg753Gln) is associated with a reduced response to bacterial lipoproteins and increased susceptibility to staphylococcal infection and tuberculosis. TLR5 detects flagellin, the major protein constituent of flagella. Approximately 10% of Caucasian individuals carry a single nucleotide polymorphism (SNP) ($TLR5^{392STOP}$) that is associated with a susceptibility to Legionnaires' disease. IL-8 is a potent chemoattractant for neutrophils. Recent studies have demonstrated that a SNP (-251 A/A genotype) in the IL-8 promoter region is associated with increased susceptibility to CDI and with increased faecal IL-8 concentrations. The aim of this study was to establish the prevalence of these TLR2, TLR5 and IL-8 SNPs within this same study population and to determine whether an association existed between these genotypes and susceptibility to CDI.

3. A recent study conducted by our group characterised clinical isolates in the Edinburgh area and found that 87% were resistant to moxifloxacin. The first aim of this study was to examine the mechanisms of fluoroquinolone resistance in this collection of isolates. It was hypothesised that the competitive advantage conferred by resistance to moxifloxacin may influence the fitness of *C. difficile* isolates, in particular growth and the expression of the virulence factors toxins A and B. Thus the second aim of this study was to determine whether a correlation exists between fitness and resistance or sensitivity to moxifloxacin.

Taken as a whole these three studies examine factors pertaining to both the host and the organism that may influence the outcome of infection of an individual by *C. difficile*.

2. Materials and Methods

2.1 Allocation of study participants to study groups

Study participants were recruited from amongst the population of colorectal patients attending a surgical outpatients' clinic or undergoing colorectal surgery at the Western General, a large Acute Edinburgh hospital. Participants were consented by Dr Surekha Reddy and each was allocated a sequential identification number of the form AW1. This number was used to identify tissue samples and faecal isolates where applicable. Participants were divided into three groups:

Cases – those individuals with clinically diagnosed CDI (toxin A/B positive stools and/or positive for PMC on colonoscopy);

Carriers - those individuals who were culture positive (toxin A/B variable) but asymptomatic;

Controls - those individuals who were culture-negative (toxin A/B negative) and asymptomatic. It should be noted that while these individuals do not have CDI they are attending the colorectal unit and are therefore not "healthy" controls.

2.1.1 Collection of faecal samples

Faecal samples were collected during clinic appointments or after admission to hospital and prior to surgery, in accordance with the terms of the consent given by the Local Research Ethics Committee.

2.1.2 Detection of toxin in stool samples

A commercial ELISA kit for the detection of toxins A and B (TechLab) was used to perform toxin assays on fresh stool samples (within 24 hours of collection) in accordance with the manufacturer's protocol.

2.1.3 Culture of stool samples and identification of *C. difficile*

Stools were cultured on Brazier's CCEY agar (Oxoid) and incubated at 37°C in anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2) for 24 to 48 hours. If no *C. difficile* colonies were seen within this period plates were incubated for a further 48 hours to confirm that the samples were culture-negative.

Preliminary identification of *C. difficile* was made on the basis of characteristic smell, colony morphology and visualisation of motility in a wet film observed by light microscopy. Examination of colonies under long wave UV light (Wood's Lamp) to check for characteristic chartreuse fluorescence was also performed. If further confirmation was required a Gram stain and PCR amplification of a fragment of the *cdd-3* gene, present in both toxigenic and non-toxigenic isolates, were performed. Isolates were stored on the bench at room temperature in cooked meat broth with cooked meat particles (Brown *et al.,* 1996).

2.2 Characterisation of faecal isolates

2.2.1 Detection of toxin production *in vitro*

If *C. difficile* was isolated from toxin-negative stools, the ability of the isolate to produce toxin was tested by growing it up in PPY (proteose peptone yeast medium) at 37°C under anaerobic conditions for 48 hours. PPY comprises 20g/L proteose peptone (Oxoid, Basingstoke, UK), 5g/L yeast extract (Oxoid, Basingstoke, UK), 5g/L trypticase (Becton, Dickson & Company, Sparks, MD), 5g/L NaCl, 0.75g/L L-cysteine-HCl, 0.4g/L Na_2CO_3, adjusted to pH 7.1. The presence of toxin in the growth medium was detected using an ELISA kit as previously described.

2.2.2 DNA extraction

Genomic DNA was extracted from cultures following the method of de Lamballerie *et al.,* (1992). Briefly, microbial cells, obtained by spinning down broth cultures or picking colonies from plates, were suspended in a 5% suspension of Chelex-100, a chelating resin (Bio-Rad Laboratories) and placed in a boiling bath. Following centrifugation, the supernatant was taken off and stored at -20°C.

2.2.3 Ribotyping

Ribotyping was performed using the method described by O'Neill *et al.,* (1996). Specific oligonucleotide primers (obtained from MWG) complementary to the 3' end of the 16s rRNA gene (positions 1445-1466) and the 5' end of the 23s rRNA gene (positions 1-20) were used to amplify the intergenic spacer region which has been shown to be very heterogeneous. Primer sequences are given in Appendix 1.

Amplification was performed in a 100µl reaction mixture containing 1x PCR buffer (10mM Tris HCl; 50mM KCl, Promega); 2.25mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 50pmol each primer; 2U of Taq (Promega) and 10µl of DNA template or, in the case of the negative control, sterile water. Cycling conditions were as follows: initial denaturation at 94°C for 4 minutes followed by 35 cycles of denaturation at 94°C for one minute; annealing at 55°C for one minute and extension at 72°C for one minute, with a final elongation step of 72°C for five minutes. The amplification products were concentrated by heating at 75°C for 100 minutes to obtain a final volume of 15µl and then run in a 3% Metaphor agarose gel (ABgene) for two and a half hours at 80 volts. Ribotypes were

determined by comparing the banding patterns obtained with those held on the laboratory database.

2.2.4 Toxinotyping

Toxinotyping was performed using the method described by Rupnik *et al.,* (1997, 1998). Fragments of the *tcdA* (A3) and *tcdB* (B1) genes were amplified by PCR and digested with restriction enzymes. The resulting restriction pattern allows determination of the toxinotype of an isolate. The primers used were obtained from VHBio and their sequences are given in Appendix 1. Amplification was performed in a 50µl reaction mixture containing 1x PCR buffer (10mM Tris-HCl; 50mM KCl, Promega); 3mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 15pmol each primer; 1U of Taq (Promega) and 5µl of DNA template or, in the case of the negative control, sterile water. The reaction mixture for the amplification of the A3 fragment also contained TMA (tetramethylammonium chloride, Sigma) with a final concentration of 10^{-3}M. Cycling conditions were as follows; initial denaturation at 93°C for three minutes followed by 30 cycles for B1 and 35 cycles for A3 of annealing and extension at 47°C for eight minutes and denaturation at 93°C for four seconds, with a final extension step at 47°C for 10 minutes. After visualisation of the fragments in 1% agarose gel, they were restricted using the enzymes *Eco*RI for A3 and *Hinc*II and *Acc*I for B1 (enzymes were obtained from New England BioLabs). Restriction was performed in a 20µl digest volume containing 1µl of the appropriate enzyme and, in the case of *Hinc*II restriction of B1, 10% BSA (Promega). Digest mixtures were incubated for two hours at 37°C and the

resulting fragments visualised in 1% agarose gel. The toxinotype of each isolate was determined as described by Rupnik *et al.,* (1997, 1998).

2.2.5 Detection of binary toxin genes

The presence of the binary toxin genes was detected following the method of Stubbs *et al.,* (2000). PCR primers were obtained from VHBio; their sequences are given in Appendix 1. Amplification was performed in a 50µl reaction mixture containing 1x PCR buffer (10mM Tris-HCl; 50mM KCl, Promega); 1.5mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 10pmol each primer; 1U of Taq (Promega) and 5µl of DNA template or, in the case of the negative control, sterile water. Cycling conditions were as follows; 30 cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for one minute and extension at 72°C for one minute 20 seconds. Products were visualised in 1% agarose gel.

2.2.6 Amplification of *cdd-3* gene

PCR amplification was performed following the method of Cohen *et al.,* (2000b); primer sequences are given in Appendix 1. Amplification was performed in a 50µl reaction mixture containing 1x PCR buffer (10mM Tris-HCl; 50mM KCl, Promega); 2.5mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 10pmol each primer; 1U of Taq (Promega) and 5µl of DNA template or, in the case of the negative control, sterile water. Cycling conditions were as follows; 40 cycles of denaturation at 95°C for one minute, annealing at 52°C for one minute and extension at 72°C for one minute, with a final extension step at 72°C for 10 minutes. Products were visualised in 1% agarose gel.

2.3 Analysis of tissue specimens

2.3.1 Immunohistochemistry

Colonic tissue specimens exhibiting no pathological changes, taken by biopsy or resection in the course of diagnosis or treatment, were obtained. The specimens had been fixed in formalin and embedded in paraffin wax.

Cut and mounted sections were dewaxed in xylene and rehydrated by passage through graded alcohols and water. Antigen retrieval was performed by treatment of sections in Tris/EDTA (pH 9) in a pressure cooker for five minutes. Sections were cooled in water for 20 minutes before two washes in PBS, each of five minutes. Sections were then treated in 3% hydrogen peroxide for 10 minutes before a further two five minute washes in PBS. Unless otherwise stated subsequent steps were performed using reagents provided in a Vectastain Universal elite ABC peroxidase kit (Vector Laboratories Inc, Burlingame, California, USA) in accordance with the manufacturer's instructions. Sections were blocked using a Vector Avidin/Biotin blocking kit (Vector Laboratories Inc), each reagent being applied for 15 minutes and sections rinsed between steps. After a five minute wash in PBS sections were blocked with normal horse serum for 10 minutes. Sections were subsequently incubated with 100µl of primary antibody, diluted in PBS, for one hour at room temperature. A negative control section was incubated with PBS in place of antibody. The primary antibodies used were specific for T cells (monoclonal mouse anti-CD3; Dako, Ely, Cambridgeshire, UK), B cells (monoclonal mouse anti-CD20; Dako), plasma cells (mouse anti-CD138; Serotec, Kidlington, Oxfordshire, UK) and macrophages (monoclonal mouse anti-CD68; Dako). Primary antibodies to CD3, CD20 and CD68 were diluted 1 in 500, while those to CD138 were diluted 1 in 1000.

After washing in PBS for five minutes sections were incubated with diluted biotinylated secondary antibody for 30 minutes. After a further five minute wash in PBS sections were incubated with Vectastain reagent for 30 minutes, then washed in PBS for five minutes. Labelled cells were visualised using DAB (Vector Laboratories Inc), the sections being incubated for 2-10 minutes at room temperature until suitable staining developed. After washing in water for five minutes sections were counterstained in haematoxylin, washed in water and dehydrated by passage through graded alcohols and xylene. Coverslips were attached and the slides allowed to dry before viewing. Counts of positively labelled cells within the lamina propria were carried out using a light microscope fitted with a stage and a 10 x 10 grid graticule in the eyepiece. Ten fields were examined for each slide and the mean count for each section calculated.

2.4 Analysis of single nucleotide polymorphisms

2.4.1 Extraction of genomic DNA from tissue sections

DNA was extracted from fixed, paraffin wax-embedded tissue sections using the method outlined by Coombs *et al.,* (1999). A 20 µm section was cut and placed in an Eppendorf tube. The tissue was deparaffinised by adding 100 µl of 0.5% Tween-20, agitating and heating to 90°C for 10 minutes on a Techne Thermal Cycler. Sections were then cooled to 55°C, the wax remaining in solution prior to digestion. This was performed by the addition of 2 µl of 10 mg/ml Proteinase K (Sigma) to give a final concentration of 200 µg/ml, followed by digestion for three hours at 55°C. Each digest solution was then heated to 99°C for 10 minutes with 100 µl of 5% Chelex-

100 suspended in Tris-EDTA. The solutions were gently shaken, centrifuged at 10,500 *g* for 15 minutes whilst still hot and then placed on ice to allow the wax to harden and be removed. The samples were then heated to 45°C and 100µl of chloroform added. After gentle agitation and centrifugation at 10,500 *g* for 15 minutes, the top phase was removed and stored at -20°C.

2.4.2 Genotyping of the IL-8 -251 allele

Genotyping was performed using a modification of the method described by Hull *et al.,* (2000). Allele-specific primers were used to identify the presence of the IL-8 -251 A and T alleles. Primers were obtained from VHBio and sequences are given in Appendix 1. Amplification was performed in a 50 µl reaction mixture containing 1x PCR buffer (10mM Tris-HCl; 50mM KCl, Promega); 1.5mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 10pmol each primer; 1U of Taq (Promega) and 2.5µl of DNA template or, in the case of the negative control, sterile water. Cycling conditions were as follows; initial denaturation at 94°C for 10 minutes followed by 35 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute, with a final extension step at 72°C for 10 minutes. Products were visualised in 1% agarose gel.

2.4.3 Genotyping of the TLR2 allele

Genotyping was performed using the method described by Ogus *et al.,* (2004). Allele-specific primers were used to identify the presence of the TLR2 2258 A and G alleles. Primers were obtained from VHBio and sequences are given in Appendix 1. Amplification was performed in a 50 µl reaction mixture containing 1x PCR buffer

(10mM Tris-HCl; 50mM KCl, Promega); 2mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 10pmol each primer; 1U of Taq (Promega) and 2µl of DNA template or, in the case of the negative control, sterile water. Cycling conditions were as follows; initial denaturation at 94°C for four minutes followed by 35 cycles of denaturation at 94°C for one minute, annealing at 62°C for one minute and extension at 72°C for one minute, with a final extension step at 72°C for four minutes. Products were visualised in 1% agarose gel.

2.4.4 Genotyping of the TLR5 allele

Genotyping was performed using the method described by Hawn *et al.,* (2003). Primers were obtained from VHBio and sequences are given in Appendix 1. Amplification was performed in a 50 µl reaction mixture containing 1x PCR buffer (10mM Tris-HCl; 50mM KCl, Promega); 1.5mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 10pmol each primer; 1U of Taq (Promega) and 2µl of DNA template or, in the case of the negative control, sterile water. Cycling conditions were as follows; initial denaturation at 94°C for five minutes followed by 30 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute, with a final extension step at 72°C for five minutes. The products of amplification were subjected to digestion with the *Dde*I restriction enzyme. Digestion was performed in a 20µl reaction mixture containing 1x enzyme buffer (Promega); 0.02µg BSA; 1µl of restriction enzyme (Promega) and 10µl of PCR product. Digest mixtures were incubated at 37°C for two hours and products were visualised in 1.8% agarose gel. The restriction enzyme cuts the variant allele only.

2.5 Moxifloxacin resistance and virulence factors

2.5.1 Isolates

Fourteen moxifloxacin-resistant and 14 sensitive isolates, as shown in Table 2.1, were selected from a collection of organisms isolated from toxin-positive stools collected between August and October 2005 from different hospitals in the Edinburgh area (Lothian University Hospitals National Health Service Trust). This collection has been previously characterised by Mutlu *et al*., (2007).

Strain	Ribotype	MIC moxifloxacin (μg ml^{-1})
EM23	001	32
EM24	001	16
EM27	001	16
EM28	001	16
EM30	001	32
EM31	001	32
EM47	070	8
EM57	106	32
EM58	106	32
EM64	106	32
EM82	106	64
EM125	106	16
EM184	001	16
EM186	001	16
EM5	023	2.5
EM14	014	2
EM36	042	2
EM83	002	1.5
EM84	070	2.5
EM87	005	1.5
EM96	005	1.5
EM102	001	1
EM109	049	1.5
EM120	002	2
EM126	005	1
EM130	106	1.5
EM169	020	2
EM185	013	0.25

Table 2.1. Characterisation of isolates (breakpoint of moxifloxacin 8 μg ml^{-1}).

2.5.2 Culture of organisms and determination of growth

The isolates were grown from spores preserved in cooked meat broth with cooked meat particles (Brown *et al.,* 1996). To prepare the starter culture, 0.5ml of the spore suspension was added to 3ml of pre-reduced PPY and incubated anaerobically overnight at 37^{o}C. The starter culture (2ml) was inoculated in 200ml of pre-reduced PPY. Ten ml of the cultures were taken every four hours for the first 16 hours, then at 24, 36 and 48 hours. Total cell counts at each time point were measured by taking aliquots of culture medium and measuring optical density (OD) at 600nm by spectrophotometer.

Cells were collected by centrifugation at 4,000 *g* for 20min, followed by 5 minutes at 9,000 *g* for maximal removal of the medium. The pellets were snap frozen in an ethanol-ice mixture and stored at -20°C prior to RNA extraction.

2.5.3 Toxin production

The amount of toxin present in the growth medium at a particular time point was determined using a commercial ELISA kit as previously described.

2.5.4 RNA extraction

RNA was extracted from pellets using a RiboPure kit (Ambion) in accordance with the manufacturer's protocol and its concentration estimated using the Nanodrop ND-1000 Spectrophotometer (Labtech). The RNA was checked for genomic DNA contamination through its use as template in a PCR reaction. RNA was stored at -70°C.

2.5.5 Reverse transcription

500ng of RNA were used as the template for cDNA synthesis using either a RetroScript (Ambion) or Superscript® VILO™ (Invitrogen) kit in accordance with the manufacturer's protocol, the products being stored at -20°C.

2.5.6 Semi-quantitative detection of gene expression

PCR primers were obtained from VHBio and sequences are shown in Appendix 1. A 2µl volume of cDNA preparations or, in the case of the negative control, sterile water was used in all amplification reactions.

Amplification of *tcdA* and *tcdB*

Fragments of the toxin A and B genes, *tcdA* and *tcdB,* were amplified separately following the method of Spigaglia & Mastrantonio (2002). Amplification was performed in a 50µl reaction mixture containing 1x buffer (10mM Tris-HCl, 50mM KCl, Promega); 2.5mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 100pmol of TA1 and TA2 primers or 25 pmol TB1 and TB2 primers and 2U of Taq (Promega). The template was denatured for 2 minutes at 95°C and then amplified for 30 cycles consisting of one minute at 95°C, one minute at 50°C and one minute at 72°C, with a final extension of 10 minutes at 72°C. Products were visualised on 1% agarose gel.

Amplification of *cme*

The *cme* gene was amplified in a 50µl reaction mixture containing 1 x buffer (10mM Tris-HCl, 50mM KCl, Promega); 2.5mM $MgCl_2$ (Promega); 200 µM each dNTP

(Amersham Pharmacia Biotech); 20pmol of forward and reverse primers and 1U of Taq (Promega). The template was denatured for three minutes at 95°C and then amplified for 30 cycles consisting of one minute at 95°C, one minute at 52°C and one minute at 72°C, with a final extension of 10 minutes at 72°C. Products were visualised in 1.5% agarose gel.

Amplification of *cdeA*

The *cdeA* gene was amplified following the method of Dridi *et al.,* (2004). Amplification was performed in a 50µl reaction mixture containing 1 x buffer (10mM Tris-HCl, 50mM KCl, Promega); 1.5mM $MgCl_2$ (Promega); 200pmol of each dNTP (Amersham Pharmacia Biotech); 20pmol of forward and reverse primers and 1U of Taq (Promega). The template was denatured for two minutes at 94°C and then amplified for 30 cycles consisting of one minute at 94°C, one minute at 55°C and one minute at 72°C, with a final extension of five minutes at 72°C. Products were visualised in 1% agarose gel.

Semi-quantitative RT-PCRs were normalised by comparison with the products of amplification of a region of the 16s rRNA gene, following the method of Nübel *et al.,* (1996). The 16s rRNA gene is transcribed at high levels throughout the exponential and stationary phases of growth. For each experiment the same cDNA preparation was used as the template for analysis of both the gene of interest and 16s expression. Primers were obtained from VHBio and used to amplify the segment from nucleotide 986 to nucleotide 1041; sequences are given in Appendix 1. Amplification was performed in a 50 µl reaction mixture containing 1x PCR buffer

(10mM Tris-HCl; 50mM KCl, Promega); 2.5mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 10pmol each primer and 1U of Taq (Promega). Cycling conditions were as follows; initial denaturation at 94°C for five minutes followed by 22 cycles of denaturation at 94°C for one minute, annealing at 63°C for one minute and extension at 72°C for one minute, with a final extension step at 72°C for five minutes. Products were visualised in 1.5% agarose gel.

2.5.7 Sequencing of the *tcdC, gyrA, gyrB* and CD3197 genes

PCR primers were obtained from VHBio and sequences are shown in Appendix 1. A 2µl volume of genomic DNA extracts (prepared as described above) was used in all amplification reactions.

A 900bp fragment of the PaLoc encompassing the *tcdC* gene was amplified with primers tcdC-F and tcdC-R. Amplification was performed in a 50 µl reaction mixture containing 1x buffer (10mM Tris-HCl, 50mM KCl, Promega); 2.5mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 10pmol of each primer and 2U of Taq (Promega). The template was denatured for two minutes at 95°C and amplified for 30 cycles consisting of one minute at 95°C, one minute at 52°C and one minute at 72°C, with a final extension of 10 minutes at 72°C. Automated Sanger sequencing was performed and fragment sequences were compared with the published sequence of the *tcdC* gene of reference strain VPI10463 using MultAlin.

The QRDR of the *gyrA* and *gyrB* genes was amplified separately with primers GyrAF and GyrAR and GyrBF and GyrBR respectively, following the method of

Drudy *et al.,* (2006). The 50µl reaction mixture contained 1x buffer (10mM Tris-HCl, 50mM KCl, Promega); 2.5mM $MgCl_2$ (Promega); 10nmol of each dNTP (Amersham Pharmacia Biotech); 10pmol of each primer and 1U of Taq (Promega). The template was denatured for three minutes at 95℃ then amplified for 30 cycles consisting of one minute at 95°C, one minute at 46℃ and one minute at 72°C. Sanger sequencing was performed and fragment sequences were compared with the published sequence of the *gyrA* and *gyrB* genes of reference strain 630 using MultAlin.

The transcriptional regulatory region CD3197 was amplified in a 50µl reaction mixture containing 1 x buffer (10mM Tris-HCl, 50mM KCl, Promega); 1.5mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 20pmol of forward and reverse primers (MerR F and MerR R) and 1U of Taq (Promega). The template was denatured for two minutes at 94°C then amplified for 30 cycles consisting of one minute at 94°C, one minute at 55℃ and one minute at 72°C. A final extension step of five minutes at 72°C was performed. The putative promoter region of CD3197 was amplified in a 50µl reaction mixture containing 1 x buffer (10mM Tris-HCl, 50mM KCl, Promega); 1.5mM $MgCl_2$ (Promega); 200µM each dNTP (Amersham Pharmacia Biotech); 20pmol of forward and reverse primers (CD3197 F and CD3197 R) and 1U of Taq (Promega). The template was denatured for two minutes at 94°C then amplified for 30 cycles consisting of one minute at 94°C, one minute at 55°C and one minute at 72°C. A final extension step of five minutes at 72°C was performed. Sanger sequencing was performed and fragment sequences were compared with the published sequence of the CD3197 region of reference strain 630 using MultAlin.

2.5.8 Analysis of amino acid sequence of GyrA, GyrB and CD3197

The amino acid sequence was derived from the nucleotide sequence using the ExPASy web tool and compared with the published sequence of reference strain 630.

2.5.9 Antibiotic susceptibility testing

MICs were determined using the agar dilution protocol given in the Clinical Laboratories Standards Institute guidelines (CLSI, 2001). Concentrations of moxifloxacin (Bayer) used were 128 - 0.5μg ml^{-1}. The isolates were subcultured from cooked meat broth into pre-reduced thioglycollate medium (Sigma) enriched with 5 μg haemin, 1 μg vitamin K_1 and 1 mg $NaHCO_3$ ml^{-1} and incubated overnight in an anaerobic chamber at 37°C. After adjusting the turbidity to 0.5 McFarland standard, aliquots (1–2 μl) of the cultures were spotted onto *Brucella* agar (Oxoid), supplemented with haemin, vitamin K_1 and 5% lysed horse blood plus moxifloxacin of a given concentration, using a multipoint inoculator and incubated anaerobically at 37°C for 48 hours. Control plates containing no antibiotic were also inoculated and incubated both anaerobically and aerobically to check for aerobe contamination. Reference strain 630 was used as a control strain as its MIC was known from a previous study (Drummond *et al.,* 2003a).

2.5.10 Single passage mutants

An overnight culture of the moxifloxacin-sensitive isolate EM96 (MIC 1.5 μg ml^{-1}) was grown up in pre-reduced thioglycollate medium as described above. 100μl of neat culture were spread onto *Brucella* agar plates, prepared as described above, plus 1.5 μg ml^{-1}, 2 μg ml^{-1} or 3 μg ml^{-1} moxifloxacin. The plates were checked after 24

and 48 hours and new colonies present at each time point were marked. After 48 hours colonies were picked and subcultured to pre-reduced thioglycollate medium overnight prior to their MICs being determined as described above. The colony with the highest MIC was identified and further passaged on plates containing increasing concentrations of moxifloxacin. This process was repeated until a moxifloxacin mutant with reduced susceptibility was obtained.

2.6 Statistics

Statistical analysis was performed using the Minitab 15 package. Data were analysed by means of the Kruskal-Wallis one-way ANOVA, MANOVA and Mann-Whitney tests. P values of ≤ 0.05 were considered statistically significant. Given the small sample size for each study population no formal statistical analysis of the genotyping data was carried out.

3. Study population and characterisation of *C. difficile* isolates

3.1 Study population

One hundred and fourteen individuals were recruited to the study, of whom 98 were recruited from among individuals attending the colorectal outpatient clinic or undergoing colorectal surgery at the Western General Hospital, Edinburgh. The remaining 16, also colorectal patients and either cases or carriers, were identified in one of three ways: clinically, on the basis of findings at colonoscopy; the submission of toxin-positive stools to the hospital's microbiology diagnostic laboratory or through culture by Dr Reddy of faecal samples submitted to the diagnostic laboratory. It is important to note that for a period of time stool samples from all patients on the colorectal surgery wards, whether symptomatic or not, were submitted for *C. difficile* testing and asymptomatic carriers were identified from these samples.

Nine carriers were identified; three from the culture of diagnostic laboratory samples and six from the colorectal clinic population. These data indicate that the carriage rate among the general population is 6% (6/98); this accords with published data reporting carriage rates in healthy adults of 3 - 7% (Kato *et al.,* 2001; Kelly & LaMont, 1998). Thirteen cases were identified, two from among the clinic population who developed CDI following admission for surgery and 11 through toxin-positive stool samples submitted to the diagnostic laboratory.

It was only possible to obtain colonic tissue samples for seven cases (including the two described above who were from the clinic population), six carriers and six controls, giving an experimental study population of 19. The mean ages of the groups were 83, 72 and 70 respectively; there was no statistically significant difference in age between the groups (p=0.31). The cases group comprised six females and one male; the carriers group five females and one male and the control group four males and two females. Although the control group is not gender matched to the other groups this is not considered of concern as there is no evidence to suggest that an association exists between gender and susceptibility to CDI.

3.2 Characterisation of isolates

Twenty three isolates of *C. difficile* from 19 of the 22 cases and carriers were characterised (Table 3.1). No organisms were isolated from two cases, clinically diagnosed, and the cooked meat broth in which an isolate from a carrier was being stored was destroyed in error. Multiple stool samples were obtained from the hospital diagnostic laboratory for three individuals; two isolates each were isolated from two individuals and three from the third.

Ribotypes

Ribotypes were identified for 18 isolates; 17 were ribotype 001 (94.4%) and one ribotype 126. Ribotype 001 was the second most common in Scotland as a whole in the three months to December 2008 among isolates typed by the Scottish Reference

Laboratory (HPS *C. difficile* Working Group, 2009a), while a study by Mutlu *et al.,* (2007) characterising clinical isolates collected in south-east Scotland in 2005 found ribotype 001 to be the most common (75.8%). All multiple isolates from individuals were found to be of the same ribotype.

Toxinotypes

Toxinotypes were identified for 22 isolates; 19 were of toxinotype 0 (86.4%) and three were variant toxinotypes, two being V and one being IV. The findings of Mutlu *et al.,* (2007) are comparable; the majority of these isolates (96.6 %) were of toxinotype 0, with two toxinotype V isolates and single isolates of toxinotypes I, IV and XIII.

Presence of binary toxin genes

The binary toxin genes, *cdtA* and *cdtB*, were found in only two isolates, both variant toxinotype V. Again these findings correspond with those of Mutlu *et al.,* (2007) who detected the binary toxin genes in three isolates: two isolates of toxinotype V and one isolate of toxinotype IV. There were no differences between the isolates obtained from carriers and cases in terms of ribotype or toxinotype.

Toxin production by isolates

In all bar one instance, a carrier, stools from which *C. difficile* was isolated were found to be positive for the presence of toxin A and/or B, as measured by ELISA. The organism isolated from this one individual was cultured *in vitro* and culture supernatant was tested for the presence of toxins; the isolate was seen to be toxigenic.

Strain	Isolated from	Ribotype	Toxinotype	*cdtA/cdtB*
AW7	Carrier	ND	0	N
AW21	Carrier	ND	0	N
AW57	Carrier	1	0	N
AW82	Carrier	ND	V	Y
AW88	Carrier	1	IV	N
AW106	Carrier	1	0	N
AW107	Carrier	1	0	N
AW108	Carrier	1	0	N
AW89	Case	1	0	N
AW95A	Case	1	0	N
AW95B	Case	1	0	N
AW96	Case	ND	0	N
AW97	Case	ND	0	N
AW98A	Case	1	0	N
AW98B	Case	1	0	N
AW98C	Case	1	ND	ND
AW99A	Case	1	0	N
AW99B	Case	1	0	N
AW100	Case	1	0	N
AW101	Case	126	V	Y
AW109	Case	1	0	N
AW110	Case	1	0	N
AW111	Case	1	0	N

Table 3.1 Characterisation of isolates. ND indicates not determined.

4. Immunohistochemical analysis of immune cell populations

4.1 Introduction

The lymphoid elements of the large intestine comprise organised lymphoid tissue including mesenteric lymph nodes and isolated lymphoid follicles. These follicles contain a core of B cells surrounded by T cells and host the induction phase of the adaptive immune response (Platt & Mowat, 2008). The effector cells of the mucosal immune system are found in the epithelium and lamina propria and include activated T cells, B and plasma cells, dendritic cells, mast cells and macrophages.

The aim of this study was to quantify and compare effector T cells, B cells, plasma cells and macrophages within the lamina propria of colonic tissue not exhibiting pathological changes taken from cases, carriers and controls. Previous research (Johal *et al.,* 2004b) reported significantly decreased numbers of IgA-secreting B/plasma cells and macrophages in cases compared with controls. Cases also showed significantly greater numbers of IgG-secreting cells. The hypothesis for the present study was that comparable immune cell counts would be seen in carriers and controls, with both being higher than those seen in cases.

4.2 Materials and methods

Colonic tissue specimens, taken by biopsy or resection in the course of diagnosis or treatment, were obtained. The specimens, which were fixed in formalin and embedded in paraffin wax, were sectioned and mounted on slides. One section from each specimen was stained with haematoxylin and eosin and examined by Dr Paul Fineron to establish that the colonic tissue present did not exhibit pathological changes.

Suitable specimens were obtained for six of the nine identified carriers and equivalent numbers of cases and controls were selected (seven cases and six controls). Sections of the specimens from these 19 individuals were incubated with cell-specific monoclonal antibodies and the cell populations were enumerated as described in section 2.3.1. It was intended that B and plasma cells would also be stained to show the class of immunoglobulin secreted. However, it proved difficult to optimise the protocol, particularly for IgA which having been secreted is found throughout tissue, thus this analysis was not pursued.

4.3 Results

Representative photographs of labelled tissue sections are shown below. Figure 4.1 shows CD3-labelled T cells, Figure 4.2 CD20-labelled B cells, Figure 4.3 CD68-labelled macrophages and Figure 4.4 CD138-labelled plasma cells.

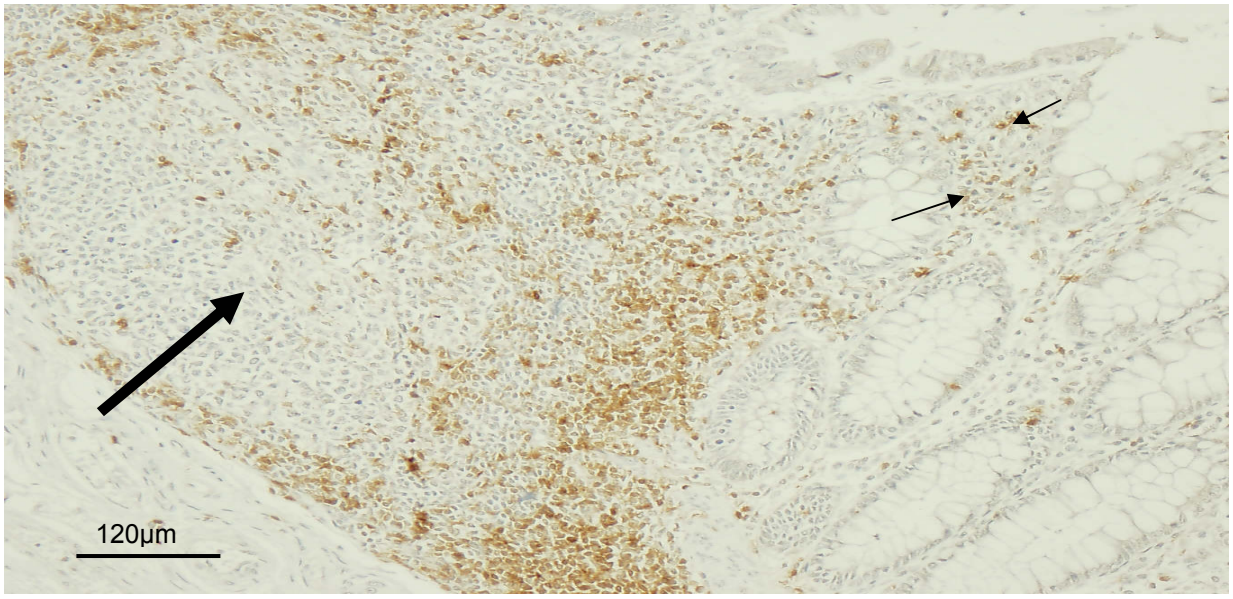


Figure 4.1. CD3-labelled T cells in a tissue specimen taken from a control. To the left of the picture T cells can be seen around the perimeter of a lymphoid follicle (large arrow). Isolated T cells in the lamina propria can be seen in the areas between glands to the right (small arrows).

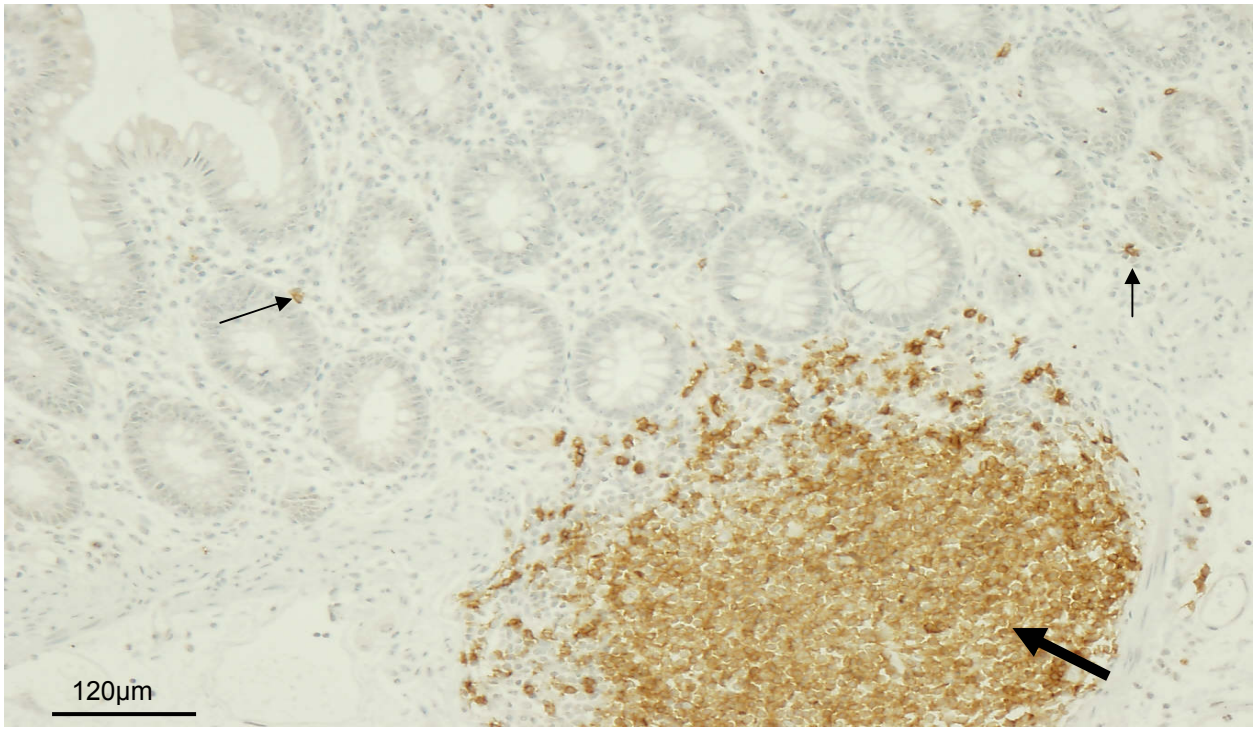


Figure 4.2. CD20-labelled B cells in a tissue specimen taken from a case. A lymphoid follicle can be seen bottom right (large arrow) with a small number of isolated cells present in the glandular region (small arrows).

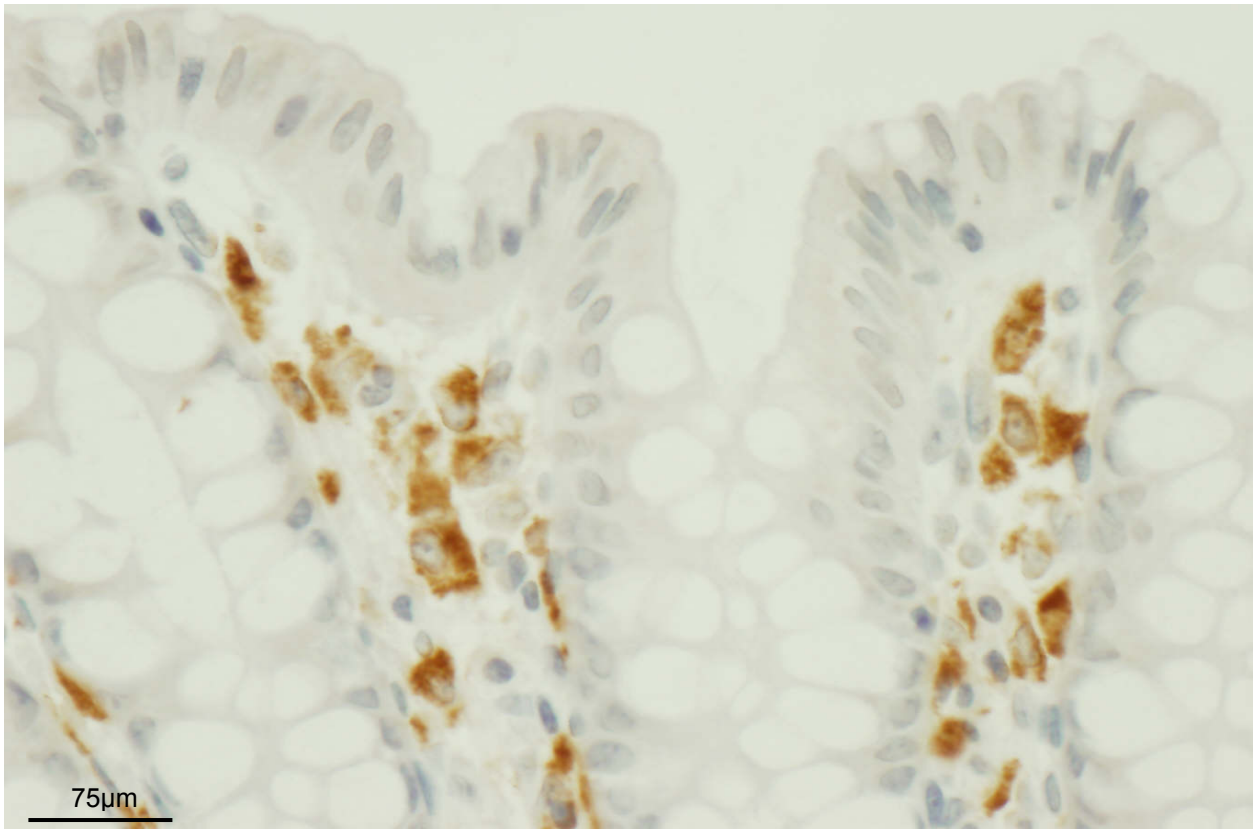


Figure 4.3. CD68-labelled macrophages in a tissue specimen taken from a control.

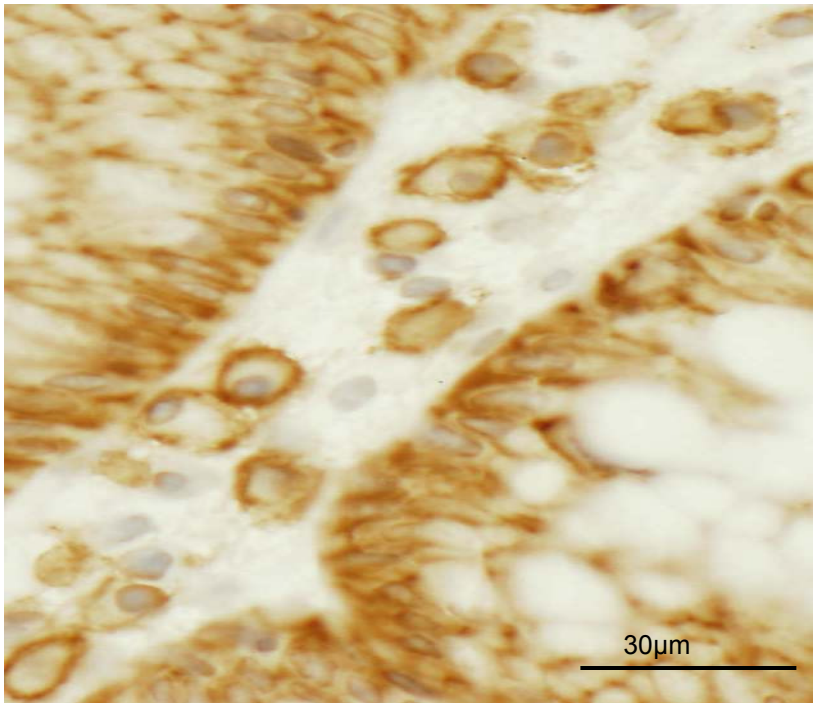


Figure 4.4. CD138-labelled plasma cells in a tissue specimen taken from a case. Epithelial cells of surrounding glands are also stained.

4.3.1 Immune cell counts

Cell counts for each study participant and cell type are given in Table 4.1.

Study Participant	Study Group	Mean count per mm^2			
		T cells	B cells	Plasma cells	Macrophages
AW96	Case	156	6	50	150
AW97	Case	75	13	*	*
AW95	Case	75	6	6	56
AW103	Case	156	19	25	219
AW81	Case	122	29	94	119
AW98	Case	56	19	125	94
AW89	Case	94	25	138	169
AW25	Control	244	25	169	356
AW6	Control	138	44	300	250
AW10	Control	63	6	56	169
AW75	Control	125	56	294	31
AW42	Control	131	13	206	213
AW4	Control	94	25	69	138
AW57	Carrier	6	0	19	56
AW82	Carrier	100	19	69	138
AW88	Carrier	188	38	38	200
AW106	Carrier	106	6	50	81
AW107	Carrier	56	6	44	63
AW108	Carrier	69	6	94	69

Table 4.1. Mean cell counts per square millimetre of colonic tissue. An asterisk indicates that a labelled section was not available for enumeration.

Analysis of macrophage counts

Figure 4.5 compares median macrophage counts for cases, carriers and controls. There is no statistically significant difference between the three groups (p = 0.22). However, it can be seen that carriers have a lower median count than both cases and controls; these differences are not significant (p = 0.32 and 0.15 respectively). Cases have a lower median count than controls, again this difference is not significant (p = 0.32).

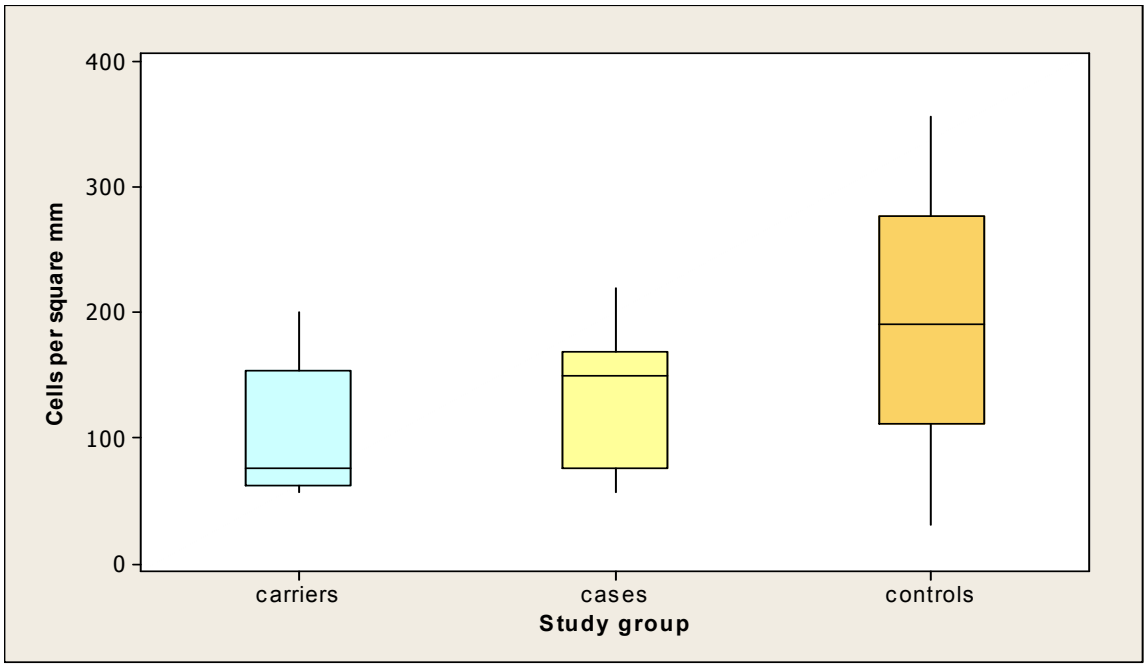


Figure 4.5. Macrophage counts per mm^2 of sectioned colonic tissue. Boxes represent the range between quartile 1 and quartile 3, with the median value represented by the horizontal line. The whiskers indicate highest and lowest values.

Analysis of T cell counts

Figure 4.6 compares median T cells counts for cases, carriers and controls. No statistically significant difference exists between the three groups (p = 0.49). Again it

can be seen that carriers have a lower median count than both cases and controls although these differences are not significant (p = 0.56 and 0.23 respectively). Cases have a lower median count than controls but this difference is not significant (p =0.7).

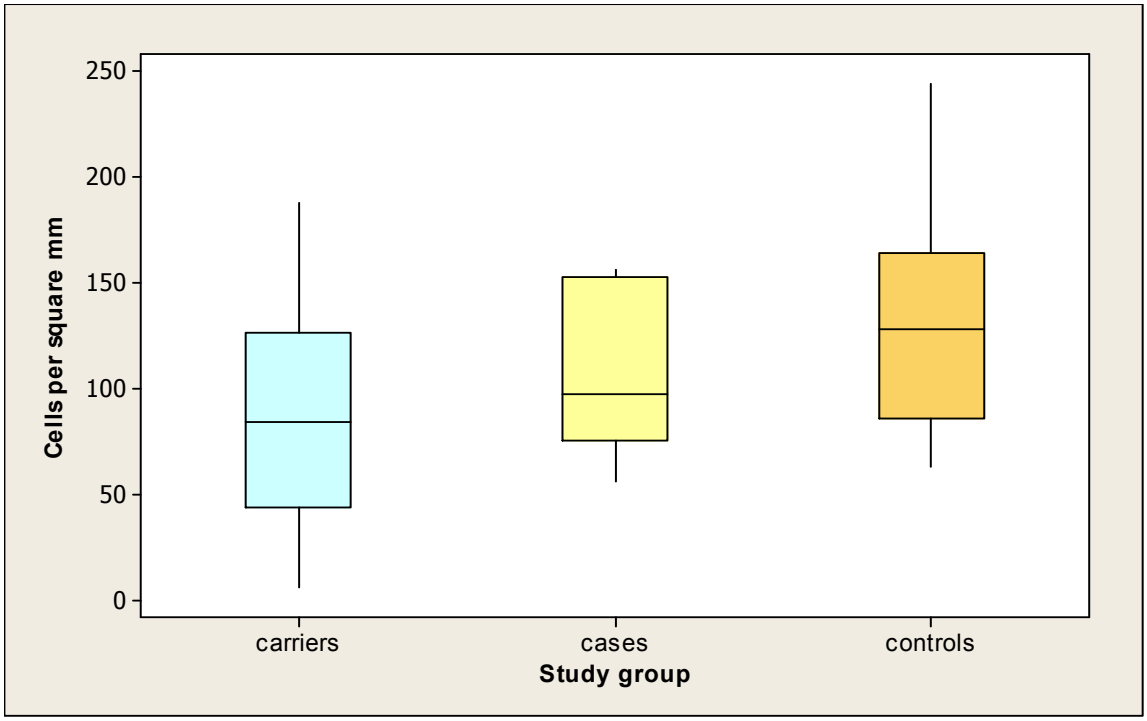


Figure 4.6. T cell counts per mm^2 of sectioned colonic tissue. Boxes represent the range between quartile 1 and quartile 3, with the median value represented by the horizontal line. The whiskers indicate highest and lowest values.

Analysis of B cell counts

Figure 4.7 compares median B cell counts for the three study groups; there is no statistically significant difference between them (p =0.16). Compared with the other cell types examined B cells are present in low numbers in all three groups, with the lowest median count being seen in the carriers. No significant difference in the median counts

exists between cases and carriers (p = 0.26) or carriers and controls (p = 0.14). While cases have a lower median count than controls again this is not significant (p = 0.29).

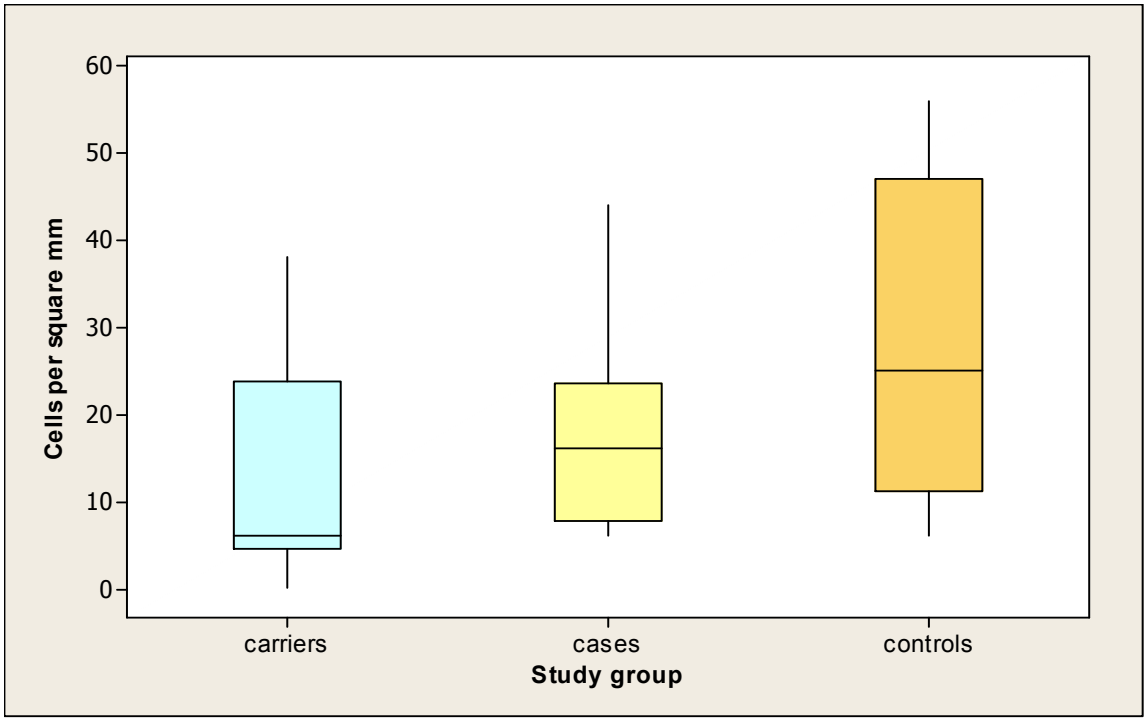


Figure 4.7. B cell counts per mm^2 of sectioned colonic tissue. Boxes represent the range between quartile 1 and quartile 3, with the median value represented by the horizontal line. The whiskers indicate highest and lowest values.

Analysis of plasma cell counts

Figure 4.8 compares median plasma cell counts for the study groups. The difference in the median counts between the three groups is statistically significant (p = 0.049). While carriers have a lower median count than cases this is not significant (p = 0.32) nor is the difference in counts between cases and controls (p = 0.13). However, the difference between carriers and controls is significant, having a p value of 0.025.

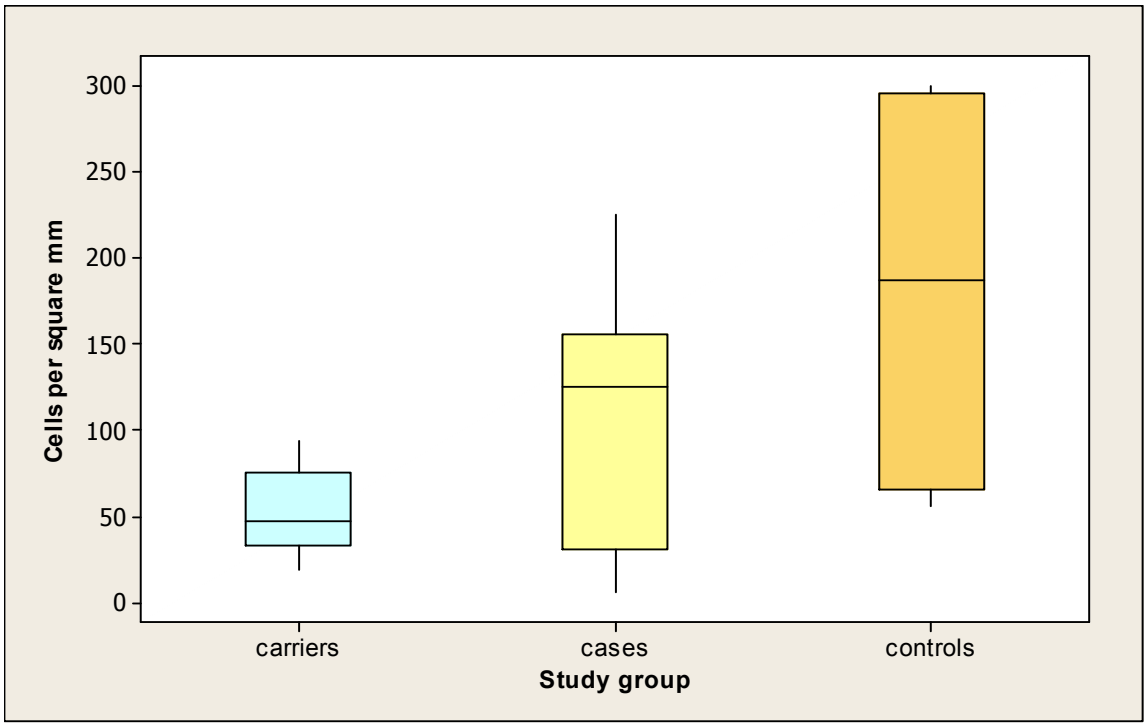


Figure 4.8. Plasma cell counts per mm^2 of sectioned colonic tissue. Boxes represent the range between quartile 1 and quartile 3, with the median value represented by the horizontal line. The whiskers indicate highest and lowest values. There is a statistically significant difference between carriers and controls (p=0.025).

Analysis of total cell counts

A multivariate analysis using the four cell counts for each individual as the vector response variable and the three study groups as the explanatory variable yielded a p-value of 0.168. Thus, this analysis provides no evidence overall for differences between the study groups in the vector of responses.

4.4 Discussion

The data derived from this pilot study support the findings of Johal *et al*., (2004b). Cases were found to have fewer of all cell types than controls although, unlike that study, these differences were not significant. However, the data do not support this study's hypothesis; carriers were found to have fewer of all four cell types examined than both cases and controls, although in only one instance was this difference significant.

These findings suggest that individuals who become infected, both symptomatically and asymptomatically, with *C. difficile* display altered mucosal immune cell populations when compared with those of uninfected individuals. With regard to cases it can be concluded that these differences pre-dated the development of symptomatic illness which occurred post-admission and resection or biopsy. However, while it is known that the two individuals from the clinic population were not infected at the time a faecal sample was taken pre-admission, the point at which they or the remaining cases became infected during admission cannot be determined. It is therefore not possible to conclude that the differences pre-dated infection. In the case of those carriers who were infected at the time faecal samples were taken at the outpatients' clinic, and therefore prior to the taking of tissue specimens, it is also not possible to determine whether these differences in cell populations pre-dated infection. Furthermore, the point at which carriers identified from culture of diagnostic laboratory samples were infected is not known. Thus on the basis of the study performed here it cannot be determined if these changes represent a predisposition to infection or have arisen as a result of exposure to the organism. It has been demonstrated that exposure of isolated colonic lamina propria cells

to high levels of toxin A *in vitro* results in macrophage loss within 72 hours (Mahida *et al.,* 1998). Exposure over longer periods of time resulted in apoptosis of T cells and eosinophils. Purified monocytes are also highly susceptible to toxin A-induced cell death, being lost much more rapidly than circulating B or T cells (Solomon *et al.,* 2005). While toxin B has not been shown to induce cell death in monocytes and macrophages it does induce rearrangement of the cytoskeleton producing morphological and functional changes (Siffert *et al.,* 1993).

The data do, however, suggest that cases and carriers mount a different immune response in the face of infection. In the case of carriers, even though the populations of effector cells are reduced, the response is protective against symptomatic infection. To understand the nature of this protective response it would be necessary to characterise the B and plasma cells in terms of the immunoglobulin class secreted and the antigen-specificity of the antibodies produced. It could be hypothesised on the basis of the findings of Johal *et al.,* (2004b) that even though cases have a greater number of plasma cells than do carriers, these are predominantly IgG-secreting, this class of immunoglobulin being less effective at protecting mucosal surfaces than IgA. The antibodies produced may also be specific for antigens that do not play a major part in pathogenesis. On the other hand, the plasma cells found in the lamina propria of carriers may be IgA-secreting and specific for *C. difficile* toxins and cell wall components such as the S-layer proteins and the adhesin Cwp66. Research into vaccine candidates has shown that immunisation of mice with a *C. difficile* cell wall extract containing these proteins protects against CDI, possibly by counteracting colonisation (Péchiné *et al.,*

2007). Characterisation of the antigen-specificity of effector $CD4^+$ T cells, particularly the T_H2 subtype, would also be required.

In conclusion, statistical analysis of the small data set reported in this study indicates that no significant differences exist in mucosal immune cell counts between the three study groups, although cell counts are seen to be lower in cases and carriers than in controls. A larger, more detailed study of the mucosal immune response would be required to cast light on the role of the host in determining the outcome of infection. A prospective study in which a series of faecal and tissue specimens were taken prior to and during hospitalisation would be most informative.

5. Genetic polymorphisms

5.1 Introduction

As mentioned in section 1.9.1 TLRs are an integral part of microbial recognition by the innate immune system.

TLR2

TLR2 plays a key role in the detection of Gram-positive organisms through its recognition of various microbial components and has been shown to interact physically and functionally with TLR1 and TLR6. TLR1 and TLR2 are co-expressed on cells of the innate immune system, including macrophages and dendritic cells (Ochoa *et al.,* 2003). This study also showed expression of both TLRs by tonsillar epithelial cells, suggesting that mucosal epithelial cells may play a role in microbial recognition. TLR1 and TLR2 appear to recognise soluble factors, porins and lipoproteins (Texereau *et al.,* 2005). Lipoteichoic acid activates immune cells via the TLR2/TLR6 heterodimer (Ozinsky *et al.,* 2000; Takeuchi *et al.,* 2001, 2002). Peptidoglycan has been reported as a TLR2 ligand, however, it has been suggested that such activity is due to the presence of lipopeptide contamination (Travassos *et al.,* 2004). A number of studies have demonstrated that TLR2-deficient ($TLR2^{-/-}$) mice are very susceptible when challenged with *Staphylococcus aureus, Streptococcus pneumoniae* and *Mycobacterium tuberculosis* (Drennan *et al.,* 2004; Echchannaoui *et al.,* 2002; Takeuchi *et al.,* 2000), illustrating the importance of TLR2 in host defence against Gram-positive and acid-fast organisms. In addition, in a model utilising TLR2 null mice, *Citrobacter rodentium*-

induced colitis is exacerbated. These mice display impaired epithelial barrier function suggesting that TLR2 signalling in immune cells may be involved in the maintenance of mucosal integrity (Gibson *et al.,* 2008; Pull *et al.,* 2005).

A polymorphism in the human *TLR2* gene (Arg753Gln) is associated with a reduced response to bacterial lipoproteins and increased susceptibility to staphylococcal infection (Lorenz *et al.,* 2000) and tuberculosis (Ogus *et al.,* 2004). In the latter study it was reported that the risk of developing TB was 6-fold higher in individuals homozygous for the variant allele and 1.6-fold higher for heterozygotes.

TLR5

TLR5 is expressed by monocytes, immature dendritic cells and on the basolateral surfaces of epithelial cells (Akira *et al.,* 2006) and detects flagellin, the major protein constituent of flagella. Approximately 10% of Caucasian individuals carry a single nucleotide polymorphism (SNP), $TLR5^{392STOP}$, that introduces a stop codon into the ligand-binding domain. This dominant mutation results in a truncated receptor with severely impaired signalling capability and is associated with a susceptibility to Legionnaires' disease (Hawn *et al.,* 2003).

IL-8

As discussed in section 1.9.2 colitis is characterised by a massive influx of neutrophils into the colonic mucosa, while the pseudomembranes of PMC are rich in neutrophils (Souza *et al.,* 1997). IL-8 is a potent chemoattractant for neutrophils and its role in the pathogenesis of CDI has been the subject of a number of studies. Steiner *et al.,* (1997)

reported that faecal IL-8 concentrations were significantly higher in patients with moderate to severe CDI than in those with mild disease. Jiang *et al.,* (2006, 2007) examined the relationship between susceptibility to CDI and genetically determined variations in the production of IL-8. These studies demonstrated that a SNP (-251 A/A genotype) in the IL-8 promoter region was associated with increased susceptibility to CDI and with increased faecal IL-8 concentrations.

The aim of this study was to establish the prevalence of these TLR2, TLR5 and IL-8 SNPs within the study population and to determine whether an association existed between these genotypes and susceptibility to CDI.

5.2 Results

DNA was extracted from paraffin wax-embedded tissue specimens using the Chelex-100/thermocycler method as described in 2.4.1. Twenty two specimens were treated as outlined, including three biopsies which were not subject to immunohistochemical staining as the architecture and/or cell composition of the tissue samples were predominantly abnormal. It was considered appropriate to use these samples as it was thought unlikely that the abnormalities seen would reflect changes in the genes under examination. The method of DNA extraction used does not have a 100% success rate; however, DNA was successfully extracted from 19 specimens, the three biopsies among them. Genotypes were determined by PCR as previously described; the results are shown in Table 5.1 and representative gels are shown in Appendix 2. In seven instances a PCR product was not obtained despite repeated attempts; the absence of a result is

indicated by an asterisk. An IL-8 genotype could not be obtained in four instances (two carriers, one case and one control); a TLR2 genotype in one instance (control) and a TLR5 genotype in two instances (one case and one control). Given the small sample size for each study population no formal statistical analysis has been carried out. The polymorphic variants are as follows: IL-8 A allele; TLR2 A allele and TLR5 T allele.

Study Participant	Study Group	Genotypes			Comments
		IL-8	TLR2	TLR5	
AW81	Case	AT	GG	*	
AW89	Case	AT	GG	CC	
AW95	Case	AT	GG	CT	
AW96	Case	AT	GG	CC	
AW97	Case	AA	GA	CT	
AW98	Case	*	GG	CC	
AW7	Carrier	AA	GG	CC	Biopsy tissue unstained
AW21	Carrier	AT	GG	CC	Biopsy tissue unstained
AW57	Carrier	TT	GG	CC	
AW82	Carrier	AT	GG	CC	
AW88	Carrier	*	GG	CC	
AW106	Carrier	*	GG	CC	
AW107	Carrier	AT	GA	CC	
AW108	Carrier	AA	GG	CC	
AW4	Control	AT	GG	CC	
AW6	Control	AT	GG	CC	
AW31	Control	AT	GG	CC	Biopsy tissue unstained
AW42	Control	*	GG	*	
AW75	Control	AT	*	CC	

Table 5.1. IL-8, TLR2 and TLR5 genotypes. An asterisk indicates that no PCR result could be obtained.

IL-8 genotypes

The variant genotype associated with increased susceptibility to CDI (-251A/A) was present in 20% of cases (1/5) and 33% of carriers (2/6). No control subjects carried this genotype. One carrier was homozygous for the wild-type allele with the remainder of the study population being heterozygous. Allele frequencies for A and T were found to be 0.6 and 0.4 in the case group, 0.58 and 0.42 in the carrier group and 0.5 and 0.5 in the control group, respectively.

TLR2 genotypes

No individuals homozygous for the variant allele were identified. Two heterozygotes were identified; one of six cases (17%) and one of eight carriers (12.5%). No control subjects carried the variant allele. Allele frequencies for G and A were found to be 0.92 and 0.08 in the case group and 0.94 and 0.06 in the carrier group, respectively.

TLR5 genotypes

Two individuals heterozygous for the dominant variant allele were identified; both were cases (2/5; 40%). The variant allele was not carried by any carrier or control subjects. The allele frequencies for C and T were 0.8 and 0.2 in the case group, respectively.

Returning to those three individuals carrying the IL-8 variant genotype associated with an increased susceptibility to CDI, it can be seen that the one case with this genotype is heterozygous for both the TLR2 and TLR5 variant alleles. The two carriers with this genotype are both homozygous for the wild-type TLR2 and TLR5 alleles.

5.3 Discussion

Given the small number of study participants it is not possible to determine whether statistically significant differences exist between the study groups in terms of genotypes. Furthermore, the small data set mandates that comparisons with published data should be considered with caution. It is, however, possible to draw inferences from the data and put forward suggestions for future research.

Of the three polymorphisms examined in this study published data regarding an association with susceptibility to CDI relate solely to the IL-8 variant genotype. In this study the variant genotype was found only in cases and carriers, being carried by 20% and 33% respectively. Jiang *et al.,* (2006) reported the presence of the variant genotype in a higher percentage of cases (39%, 15/38) and among the control group (16%, 12/73). Allele frequencies in this study and that of Jiang *et al.,* were, however, very little different. Frequencies for A and T in the case group were 0.6 and 0.4 respectively, in both studies. In the control group frequencies were 0.5 and 0.5 in this study and 0.49 and 0.51 in that of Jiang *et al.* The study by Jiang *et al.,* did not look at carriers but the authors hypothesised that IL-8 genotype would not relate to asymptomatic infection. The data presented here would suggest that this may not be the case given that the variant genotype was more prevalent in the carrier group and allele frequencies at 0.58 and 0.42 differ very little from those of the case group. The finding of a comparable prevalence of the variant genotype among carriers suggests that additional impairments of the host immune response must prevail within an infected individual for symptoms to develop. In their second study Jiang *et al.,* (2007) examined the relationship between IL-8

polymorphism and serum levels of IgG anti-toxin A in CDI cases and controls. They reported that individuals with CDI carrying the IL-8 A/A genotype were significantly more likely to show an impaired humoral immune response to toxin A than those individuals with CDI not carrying this genotype. In the present study it was found that the one case carrying the IL-8 -251 A/A genotype was heterozygous for the TLR5 dominant variant allele while the two carriers with this genotype were both homozygous for the wild-type TLR5 allele. Furthermore, the data suggest that the presence of the TLR5 variant allele alone may predispose to CDI given that the second case carrying this variant carried the IL-8 -251 A/T genotype.

The data presented here show that the TLR2 variant A allele has a low frequency within the study population as a whole. The study by Ogus *et al.,* (2004) reported that the A allele was present in 17.9% of TB patients and 7.7% of controls. Allele frequencies for G and A were found to be 0.86 and 0.14 among TB cases and 0.95 and 0.05 among controls. Allele frequencies identified within the present study are much closer to those of the Ogus control group. The two individuals carrying the variant allele, both heterozygotes, were a case and a carrier suggesting the possibility that the polymorphism is associated with infection but not necessarily symptomatic disease.

Given that the polymorphisms under investigation are relatively rare a much larger study population than that examined here would be required to ensure sufficient power to detect statistically significant differences between the study groups. The data presented here show that variant genotypes were found only in cases and carriers although the

sample size is too limited to permit inferences to be drawn about association of the variants with susceptibility to CDI.

6. Mechanisms of moxifloxacin resistance and investigation of the impact of resistance on bacterial fitness

6.1 Introduction

The use of fluoroquinolones in clinical practice is widespread and it has been suggested that their use in hospitals could be increasingly responsible for outbreaks of CDI for two reasons (Gerding, 2004). Firstly, the newer fluoroquinolones have greater activity against anaerobes and are therefore more likely to disrupt normal flora. Secondly, they may exert selective pressure favouring the emergence of fluoroquinolone-resistant strains during antibiotic treatment and the subsequent proliferation of resistant isolates. Moxifloxacin resistance has been found to be prevalent in clinical strains of *C. difficile* in the Edinburgh area of Scotland; a recent characterisation study by our group found that 87% of the strains tested were resistant to this antibiotic (Mutlu *et al.,* 2007). Resistance to fluoroquinolones is mediated by different mechanisms including mutations of DNA gyrase (the subunits of which are encoded by *gyrA* and *gyrB)* and topoisomerase IV genes (*parC* and *parE*), reduced permeability and the presence of efflux pumps (Higgins *et al.,* 2003). Analysis of its genome has shown that *C. difficile,* in common with organisms such as *Treponema pallidum* and *Helicobacter pylori,* lacks the genes for topoisomerase IV (Dridi *et al.,* 2002). Fluoroquinolone resistant isolates of

C. difficile characterised to date have shown amino acid changes in the quinolone resistance determining region (QRDR) of GyrA and GyrB. Efflux pumps are involved in antimicrobial resistance in many species of bacteria, however, as discussed in section 1.10, little is known as yet about the role of efflux pumps in multidrug resistance in *C. difficile.* Two genes have been shown to confer to resistance to certain chemical compounds: *cdeA* (*C. difficile* efflux), having homology to members of the MATE (multidrug and toxic compound extrusion) family of efflux pumps (Dridi *et al.,* 2004); and *cme* (*Clostridium* multidrug efflux), homologous to members of the major facilitator superfamily (Lebel *et al.,* 2004).

Immediately upstream of *cme* in the *C. difficile* genome is a gene designated CD3197, a putative MerR family transcriptional regulator. Members of this family have been shown to activate suboptimal sigma 70-dependent promoters in which the -35 and -10 elements are separated by more than the optimal 17+/-1 bp (Brown *et al.,* 2003). Transcription is activated through protein-mediated DNA distortion and the majority of MerR regulators respond to environmental stressors such as the presence of antibiotics.

6.2 Aims

Given the possible importance of fluoroquinolone resistance in the aetiology of CDI the first aim of this study was to examine the mechanisms of moxifloxacin resistance in our collection of clinical strains (Mutlu *et al.,* 2007) and also in a moxifloxacin mutant, with reduced susceptibility, derived from a sensitive parent strain. The study focused on the

analysis of the *gyrA* and *gyrB* genes and looked in greater depth than previous studies at a possible role for *cme* and *cdeA* by examining gene expression. Overexpression of such genes may result from the presence of mutations within the promoter region of the genes' transcriptional regulator(s) or within the regulator(s) themselves. Therefore CD3197, a putative regulator of *cme,* and its promoter region were also examined.

It was hypothesised that the competitive advantage conferred by resistance to moxifloxacin may influence the fitness of *C. difficile* strains, in particular growth and the expression of the virulence factors toxins A and B. Thus the second aim of this study was to determine whether a correlation exists between fitness and resistance or sensitivity to moxifloxacin.

6.3 Materials and methods

Twenty eight strains, 14 resistant and 14 sensitive, were selected from the strain collection and MICs confirmed by the agar dilution method. The *gyrA, gyrB* and *tcdC* genes were analysed by PCR amplification and DNA sequencing. Toxin production was examined by means of semi-quantitative RT-PCR amplification of *tcdA* and *tdcB*; all such RT-PCRs were normalised by comparison with the products of amplification of a region of the 16s rRNA gene, which is transcribed at high levels throughout the exponential and stationary phases of growth. For each experiment the same cDNA preparation was used as the template for analysis of both the gene of interest and 16s expression. Toxin production was further examined by measurement of toxin

concentration in the growth medium by ELISA. The data from pilot experiments, reported in section 6.4.3, identified 16 hours (early/mid stationary phase) and 24 hours (late stationary phase) post-inoculation as appropriate time points for analysis.

The roles of *cme* and *cdeA* were examined by analysing gene expression through semi-quantitative RT-PCR. The time points of 16 and 24 hours post-inoculation were again chosen for analysis following pilot experiments reported in section 6.4.2. CD3197 and its putative promoter region were amplified by PCR for a selection of strains, sequenced and compared with those of reference strain 630. All procedures are described more fully in Chapter 2.

6.4 Results

Mechanisms of moxifloxacin resistance

6.4.1 MICs and *gyrA* and *B* sequencing

MICs for moxifloxacin were determined for 28 strains and are shown in Table 6.1. All resistant (MIC $\geq$ 16 μg ml^{-1} **)** strains tested, with the exception of EM47 which has intermediate resistance, carry the common *gyrA* mutation ACT→ATT (Thr82→Ile) (Ackermann *et al.,* 2001). This mutation is not present in any sensitive strains. EM47 carried no other *gyrA* mutation. No mutations in *gyrB* were found in any strain.

Strain	Ribotype	MIC (μg ml^{-1})	*gyrA* mutation	*gyrB* mutation
EM23	001	32	Thr82→ Ile	N
EM24	001	16	Thr82→ Ile	N
EM27	001	16	Thr82→ Ile	N
EM28	001	16	Thr82→ Ile	N
EM30	001	32	Thr82→ Ile	N
EM31	001	32	Thr82→ Ile	N
EM47	070	8	N	N
EM57	106	32	Thr82→ Ile	N
EM58	106	32	Thr82→ Ile	N
EM64	106	32	Thr82→ Ile	N
EM82	106	64	Thr82→ Ile	N
EM125	106	16	Thr82→ Ile	N
EM184	001	16	Thr82→ Ile	N
EM186	001	16	Thr82→ Ile	N
EM5	023	2.5	N	N
EM14	014	2	N	N
EM36	042	2	N	N
EM83	002	1.5	N	N
EM84	070	2.5	N	N
EM87	005	1.5	N	N
EM96	005	1.5	N	N
EM102	001	1	N	N
EM109	049	1.5	N	N
EM120	002	2	N	N
EM126	005	1	N	N
EM130	106	1.5	N	N
EM169	020	2	N	N
EM185	013	0.25	N	N

Table 6.1. Characterisation of strains. (N = no mutation present). Breakpoint for moxifloxacin 8μg ml^{-1}.

Figure 6.1 shows the nucleotide (a) and amino acid sequence (b) of the amplified fragment of *gyrA* for EM28 (resistant) and EM96 (sensitive) aligned with those of 630. The presence of the ACT→ATT (Thr82→Ile) mutation is highlighted.

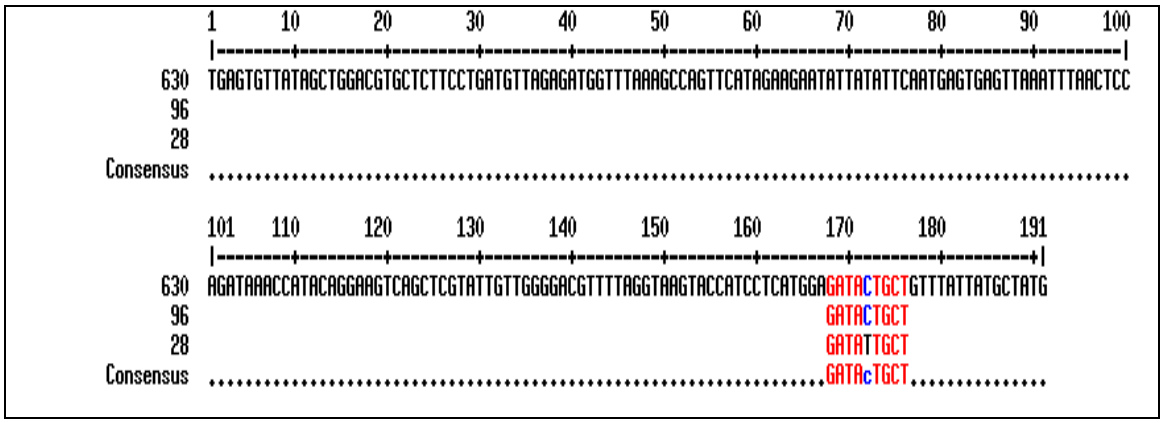


Figure 6.1 (a). Nucleotide sequence of sense strand *gyrA* fragment of EM28 and EM96 aligned with that of 630 (sensitive). Fragment comprises nucleotides 74-264 of *gyrA*. The ACT→ATT mutation present in EM28 is highlighted at position 172.

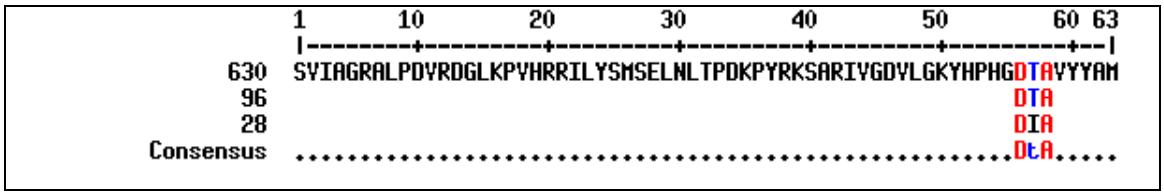


Figure 6.1 (b). Amino acid sequence of GyrA fragment of EM28 and EM96 aligned with that of 630. Fragment comprises residues 26-88. The Thr→Ile mutation present in EM28 is highlighted at position 57.

Single passage mutant

A moxifloxacin mutant of a sensitive parent strain was isolated as follows. An overnight culture of EM96 (MIC 1.5 µg ml^{-1}) was grown up in pre-reduced thioglycollate medium as described in section 2.5.10. 100µl of neat culture were spread onto *Brucella* agar plates containing 1.5 µg ml^{-1}, 2 µg ml^{-1} or 3 µg ml^{-1} moxifloxacin. After 48 hours colonies were picked and subcultured to pre-reduced thioglycollate medium overnight

prior to their MICs being determined. The colony with the highest MIC was identified and further passaged on plates containing increasing concentrations of moxifloxacin. An isolate designated EM96-R with an MIC of 6.5µg $ml^{-1}$was isolated. Sequencing of *gyrA* and *gyrB* genes of EM96-R did not reveal the presence of the Thr82→Ile mutation in *gyrA* nor any common mutations in *gyrB.* Analysis of the amino acid sequences of the GyrA and GyrB proteins revealed no differences between the parent, 630 and EM96-R.

6.4.2 Investigation of possible role of efflux pumps *cdeA* and *cme* in moxifloxacin resistance

Expression of *cdeA*

EM28, EM125, EM47, EM96 and EM96-R were grown up on two separate occasions, as described in section 2.5.2. Semi-quantitative RT-PCR products were present at 16 hours post-inoculation for only one strain, EM28, in one experiment only (Figure 6.2, lane 8), suggesting that this gene is not involved in resistance to moxifloxacin in this group of strains.

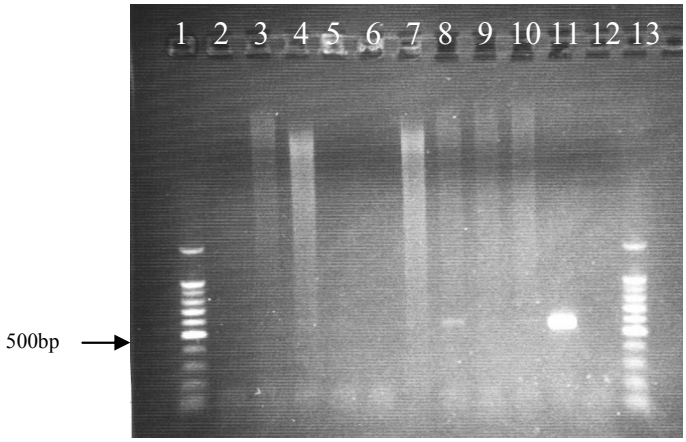


Figure 6.2. RT-PCR amplification of *cdeA* gene, fragment size 560bp. Lanes 1 and 13 100bp ladder (Promega). Lane 2 EM96-R (int), lane 3 EM96 (S), lane 4 EM47 (int), lane 5 EM28 (R), lane 6 EM125 (R), lane 7 EM96-R (int), lane 8 EM28 (R), lane 9 EM47 (int), lane 10 EM125 (R), lane 11 630 gDNA (positive control), lane 12 negative control.
Int = intermediate susceptibility, S=sensitive, R=resistant.

Expression of *cme*

Pilot semi-quantitative RT-PCR experiments with EM28 (resistant) and EM102 (sensitive) examining expression of this gene at 16 hours (early/mid stationary phase) and 24 hours (late stationary phase) post-inoculation indicated that identifiable differences existed between the two strains. At 16 hours expression by EM28 was higher than that seen in EM102; by 24 hours reduced expression by EM28 was seen, equivalent to that seen in EM102 (Figure 6.3a). Analysis of expression was normalised by examination of 16s gene expression; a representative gel of this standard is shown as Figure 6.3d confirming that equivalent levels of cDNA are present for each strain at each time point. These time points were utilised in subsequent experiments. The pattern of expression seen in the pilot experiment was not replicated in two further experiments. In the first (Figure 6.3b) no expression was seen for either strain at both 16 and 24 hours post-inoculation and in the second (Figure 6.3c) expression was seen only for EM28 at 16 hours post-inoculation, as evidenced by the 198bp band in lane 6.

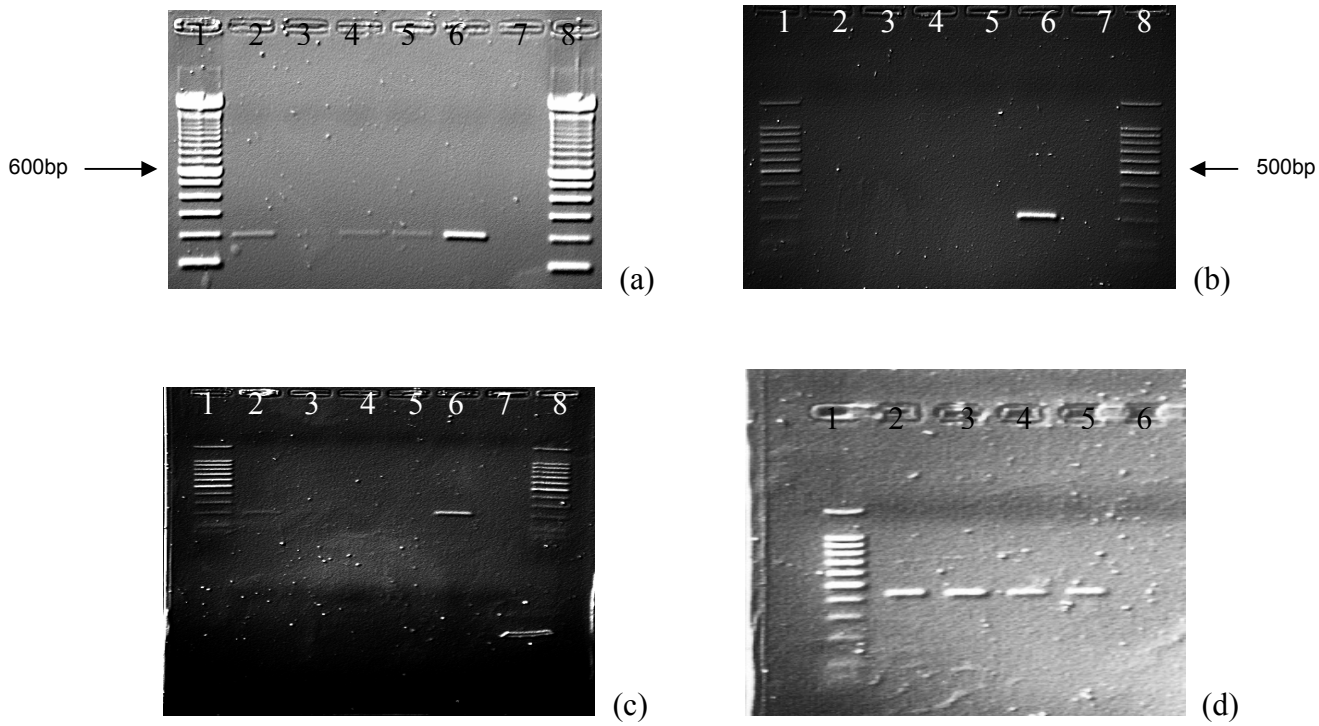


Figure 6.3 (a), (b), (c). RT-PCR amplification of *cme* gene, fragment size 198 bp. Lanes 1 and 8 100bp ladder (Invitrogen or Promega), lane 2 EM28 at 16 hours, lane 3 EM102 at 16 hours, lane 4 EM28 at 24 hours, lane 5 EM102 at 24 hours, lane 6 630 gDNA, lane 7 negative control.

(d) representative 16s standards for experiment (c), fragment size 433bp. Lane 1 100bp ladder; lane 2 EM28 at 16 hours; lane 3 EM102 at 16 hours; lane 4 EM28 at 24 hours; lane 5 EM102 at 24 hours; lane 6 negative control.

In order to obtain a clearer picture expression in a larger selection of strains was examined. Figure 6.4 shows expression at 16 hours post-inoculation in six strains (three resistant and three sensitive including EM28 and EM102). The data suggest that expression in two of the three sensitive strains (lanes 3, 4 and 5) was lower than that seen in the resistant strains (lanes 2, 6 and 7).

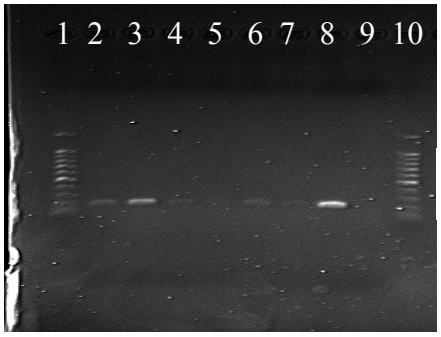
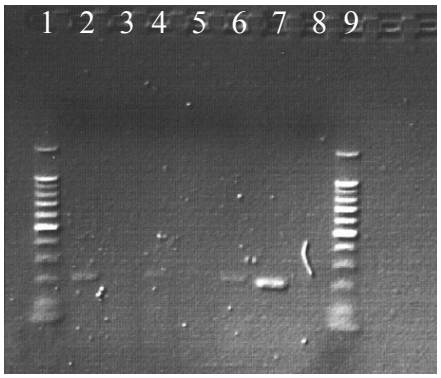
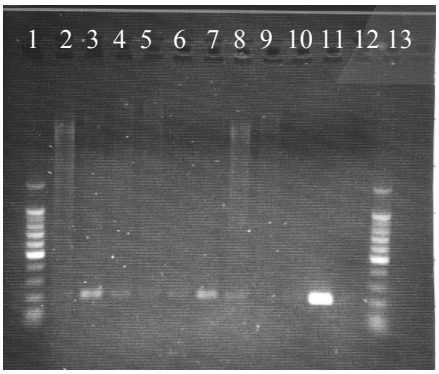


Figure 6.4. RT-PCR amplification of *cme* gene fragment. Lanes 1 and 10 100bp ladder (Promega). Lane 2 EM28, lane 3 EM96 (S), lane 4 EM102, lane 5 EM120 (S), lane 6 EM125 (R), lane 7 EM184 (R), lane 8 630 gDNA, lane 9 negative control.

Figure 6.5 shows expression in EM28, EM125, EM96 and also EM47 (intermediate resistance) and EM96-R in two separate experiments. As in previous experiments, expression was seen for EM28 and EM125. A level of expression comparable to these resistant strains was seen for EM47 but no expression was detectable for EM96-R or its parent EM96.



(a)



(b)

Figure 6.5. RT-PCR amplification of *cme* gene fragment in two separate experiments.

(a) Lanes 1 and 9 100bp ladder (Promega). Lane 2 EM28 (band present), lane 3 EM96, lane 4 EM125 (band present), lane 5 EM96-R, lane 6 EM47 (band present), lane 7 630 gDNA, lane 8 negative control.

(b) Lanes 1 and 13 100bp ladder (Promega). Lane 2 EM96-R, lane 3 EM28 (band present), lane 4 EM125 (band present), lane 5 EM47 (band present), lane 6 EM125 (band present), lane 7 EM28 (band present), lane 8 EM47 (band present), lane 9 EM96, lane 10 EM96-R, lane 11 630 gDNA, lane 12 negative control.

Analysis of the MerR family transcriptional regulator CD3197 and its putative promoter region

The semi-quantitative analysis of *cme* expression described above suggested a possible association between overexpression of this gene and reduced susceptibility to moxifloxacin. Expression of efflux pumps under normal growth conditions is tightly controlled in the absence of mutations in their regulatory mechanisms. Analysis of the putative transcriptional regulator of *cme*, CD3197, and its promoter region (Figure 6.6) was therefore undertaken to determine if any such mutations could be identified and whether their presence was associated with overexpression of *cme.*

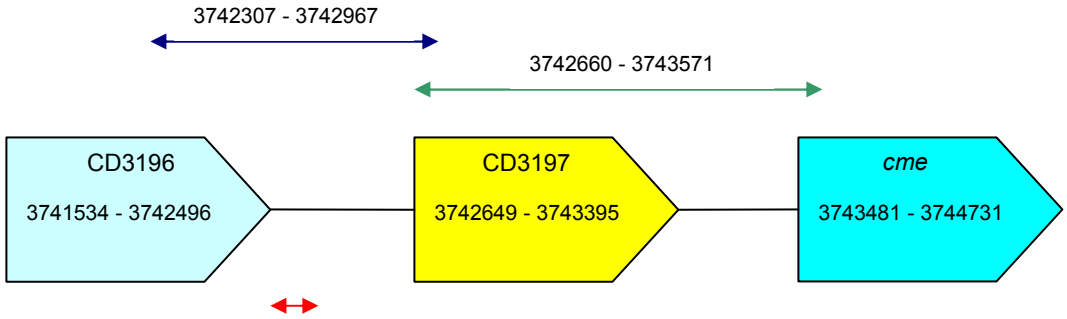


Figure 6.6. Diagrammatic representation of the *cme* locus in *C. difficile* 630. CD3197 is the putative regulator of *cme*, the efflux pump gene. Coloured boxes indicate direction of transcription. Nucleotide regions sequenced are indicated by blue and green arrows. The region in which repeat unit insertions and deletions are found is indicated by a red arrow.

The locus was amplified and sequenced for seven moxifloxacin resistant and five sensitive strains, together with EM47 and EM96-R. Nucleotide and amino acid sequences of the amplified fragments were compared with those of reference strain 630. In all strains the CD3197/*cme* region did not differ from that of the reference strain,

however, upstream of CD3197 three genotypic variants were identified: a 95p insertion, a 19bp deletion and a 38bp deletion, illustrated in Figures 6.7, 6.8 and 6.9. The deletions and insertion involve a 19bp forward repeat sequence (TCATAAACTAATTACTCTT) that appears three times in tandem in the genome of strain 630, 25bp downstream of the start of the -10 promoter element and 145bp upstream of the open reading frame of CD3197. A forward repeat is a DNA fragment appearing twice or more in the same orientation and on the same strand. In the case of the 95bp insertion the sequence is repeated in tandem four times in full and twice partially (Figure 6.8b).

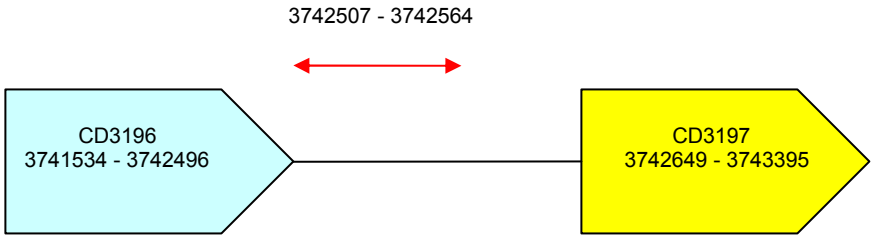


Figure 6.7. Diagrammatic representation of promoter repeat region, indicated by a red arrow.

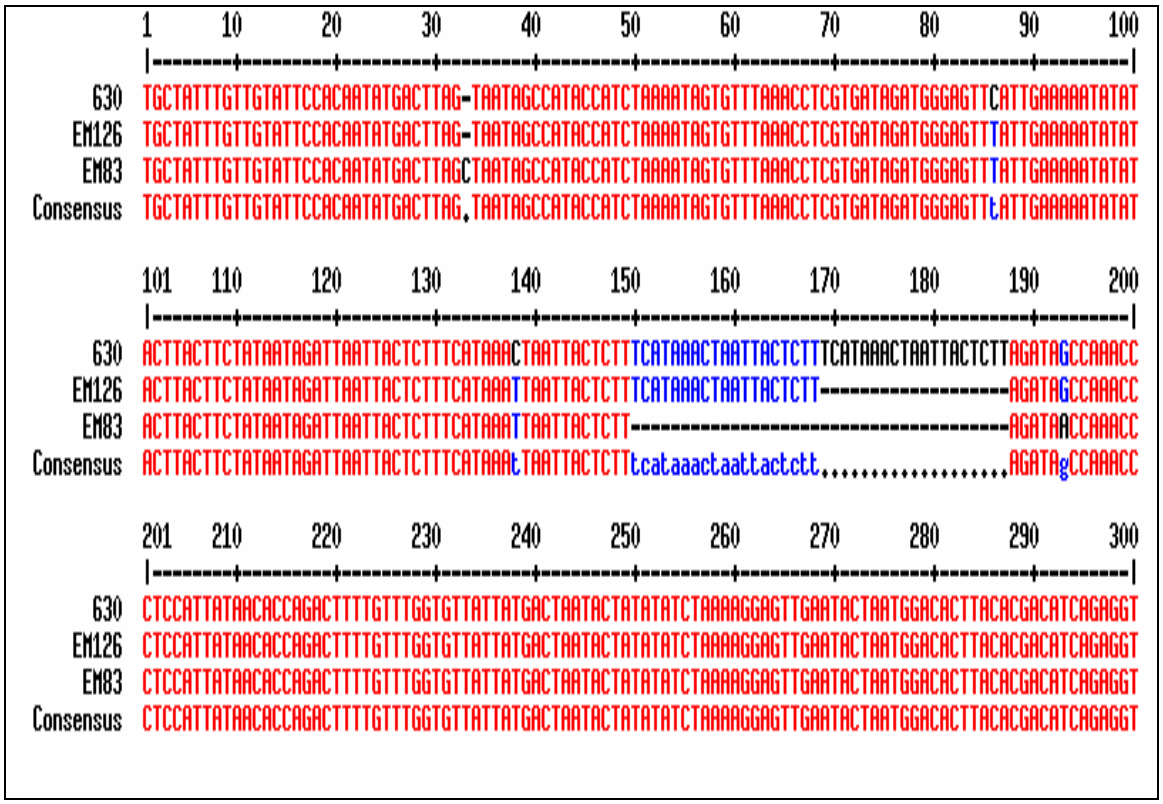


Figure 6.8a. Nucleotide sequences of the CD3197 promoter region of EM83 and EM126 compared with that of reference strain 630 (nucleotides 3742376 – 3742674). Three tandem repeats of the 19bp sequence are found in 630 between nucleotides 131 and 187 (underlined). Two repeats are found in EM126 and one in EM83. The putative -35 and -10 promoter sequences begin at positions 88 and 107 respectively (underlined). The open reading frame of CD3197 starts at position 275 (marked with an arrow).

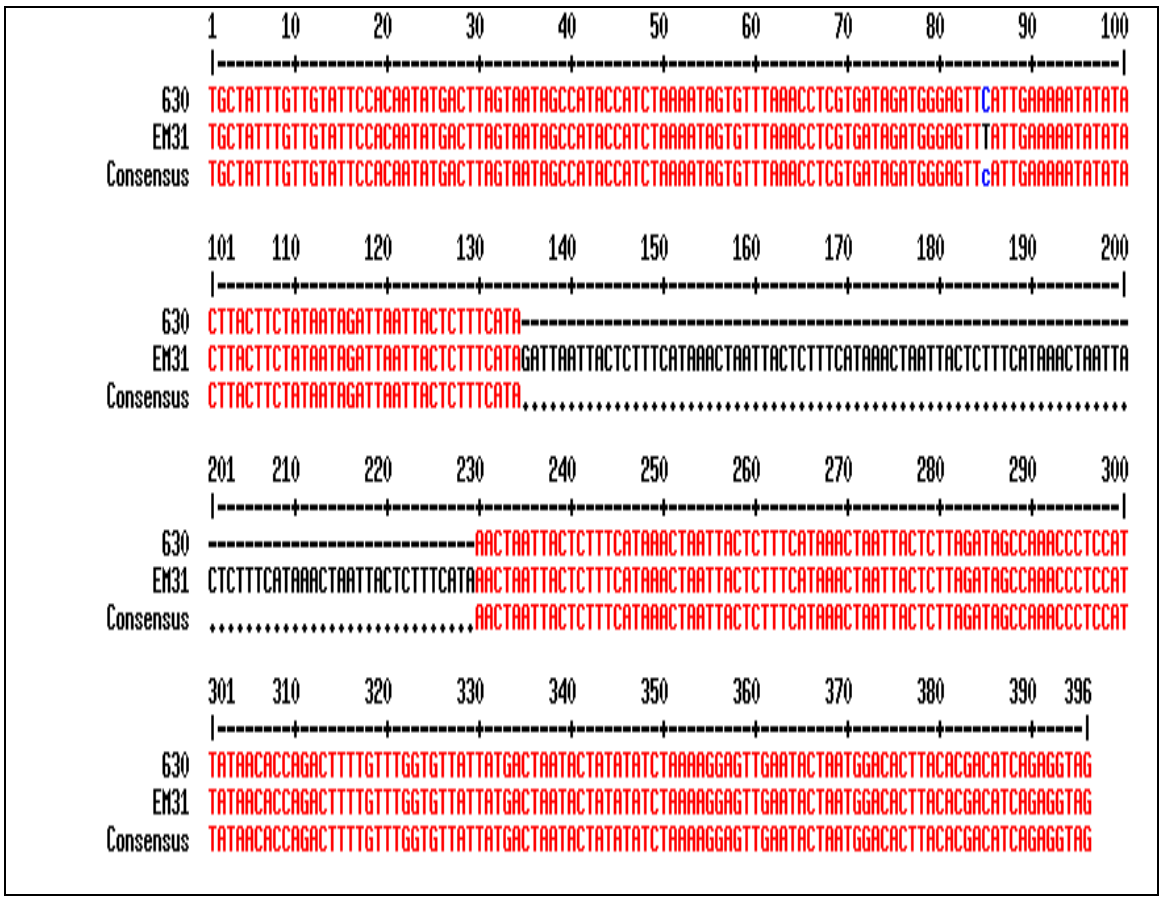


Figure 6.8b. Nucleotide sequence of the CD3197 promoter region of EM31 compared with that of reference strain 630 (nucleotides 3742376 – 3742676), showing 95bp insertion. The three tandem repeats present in 630 are found between nucleotides 130-134 and 230-281 (underlined). The putative -35 and -10 promoter sequences begin at positions 87 and 106 respectively (underlined). The open reading frame of CD3197 starts at position 369 (marked with an arrow).

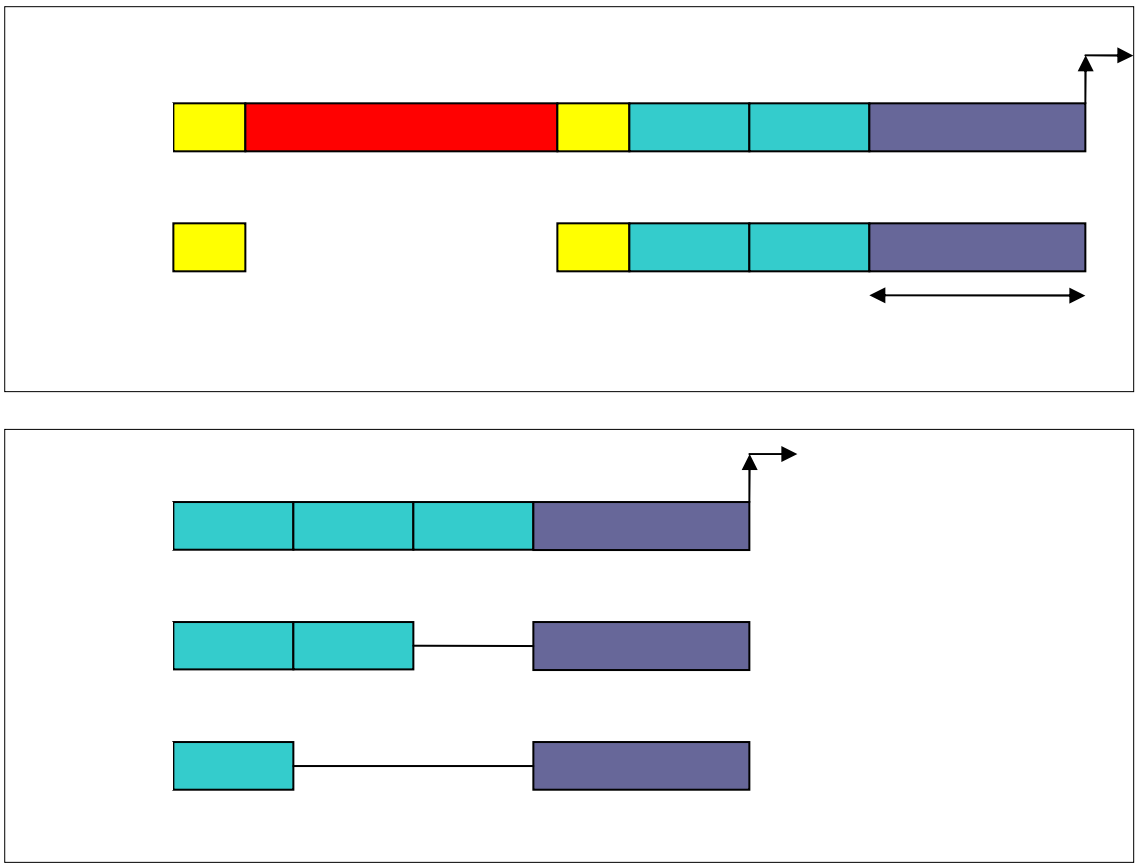


Figure 6.9. Diagrammatic representation of repeat sequence insertions and deletions in the promoter region of CD3197. Yellow boxes – partial repeats; turquoise boxes – complete 19bp repeats; red box – 95bp insertion of repeat sequences; deletions are indicated by a black line. The start of the ORF for CD3197 is indicated by an arrow.

A BLAST analysis of the 19bp repeat sequence, using low-stringency parameters, did not identify any homologous sequences in other bacterial genomes.

The distribution of the variants among the strains is outlined in Table 6.2.

Strain	Moxifloxacin susceptibility	Ribotype	Variant
630	Sensitive	012	Wild-type (three tandem repeats)
EM31	Resistant	001	95bp insertion
EM102	Sensitive	001	95bp insertion
EM24	Resistant	001	95bp insertion
EM28	Resistant	001	95bp insertion
EM83	Sensitive	002	38bp deletion
EM126	Sensitive	005	19bp deletion
EM96-R	Intermediate	005	19bp deletion
EM96	Sensitive	005	19bp deletion
EM64	Resistant	106	Wild-type
EM84	Sensitive	070	Wild-type
EM57	Resistant	106	Wild-type
EM82	Resistant	106	Wild-type
EM47	Intermediate	070	Wild-type
EM125	Resistant	106	Wild-type

Table 6.2. Prevalence of promoter region variant genotypes among sequenced strains. EM96-R is a moxifloxacin mutant derived from EM96.

EM96-R and its parent, EM96, carry the 19bp deletion, suggesting that the selective pressure exerted by moxifloxacin does not impact on this region of the genome.

Semi-quantitative analysis of *cme* expression was undertaken for those sequenced strains not previously examined (data not shown). The sequencing results and all expression

data obtained do not support an association between the presence of a variant genotype and overexpression of *cme*. Of those strains carrying a variant genotype only EM28 has been shown to overexpress this gene. Furthermore the data obtained from analysis of this further group of strains do not support a role for *cme* in moxifloxacin resistance as no differences in expression were seen between the resistant and sensitive strains. Given the limited number of strains tested these findings may reflect a type II statistical error, i.e. the sample size lacks sufficient power to detect a statistically significant difference.

Moxifloxacin resistance and fitness

6.4.3 Analysis of toxin production

tcdC sequencing

As discussed in section 1.8.4, TcdC is a negative regulator of *tcdA* and *tcdB* expression. Mutations in *tcdC* have been identified that may alter the protein's functionality resulting in overexpression of toxins. To exclude the possibility that such mutations were influencing toxin production by the strains under examination the sequenced fragments were compared with that of reference strain VPI10463. Readable sequences were obtained for 24 strains. Repeated attempts were made to obtain sequences for all strains, however, cost and time constraints meant that was not achieved. Sequences were not obtained for EM5, EM14, EM36 and EM120, all sensitive strains. Analysis of the sequence of the amplified fragments showed that none of the most common variant

genotypes identified (Curry *et al.,* 2007; Spigaglia & Mastrantonio, 2002) was present in any strain. Figure 6.10 shows a representative sequence, from EM83, aligned with that of VPI10463.

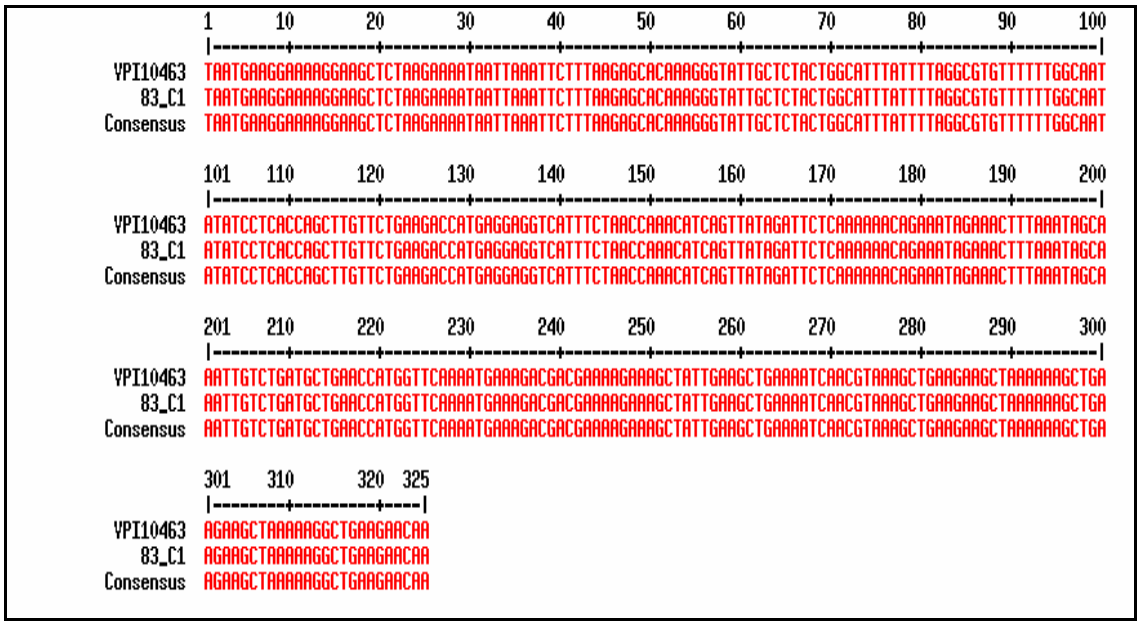


Figure 6.10 (a). Nucleotide sequence of *tcdC* fragment of EM83 aligned with that of VPI10463. Only that region in which mutations have been reported is shown (nucleotides 36-360).

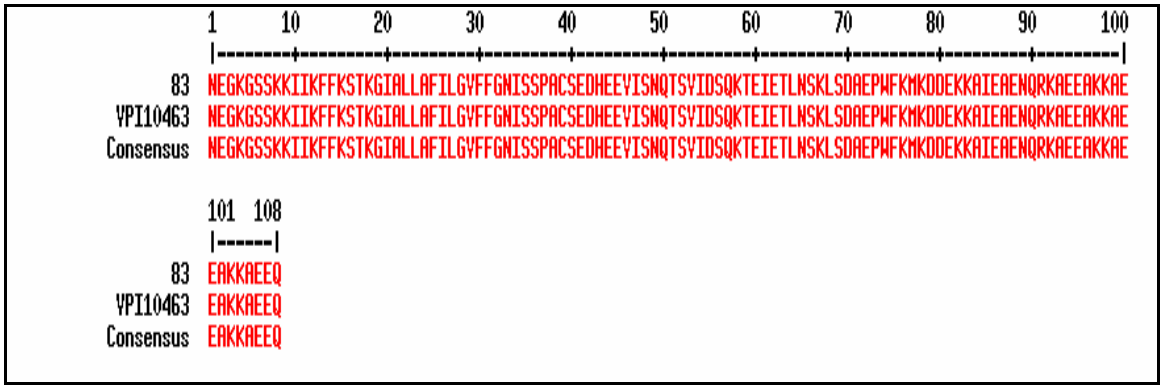


Figure 6.10 (b). Amino acid sequence of *tcdC* fragment of EM83 aligned with that of VPI10463 (residues 13-120).

Growth curves

Pilot experiments were carried out using one resistant strain (EM28) and one sensitive strain (EM102) to determine appropriate time points for sampling. Figure 6.11 shows the growth curve of the two strains over a 48 hour period expressed as OD values; little difference in the rate of growth or maximum OD was observed between the two strains. OD measured at 600nm has been shown previously to correlate with viable counts (Drummond & Poxton, 2003b).

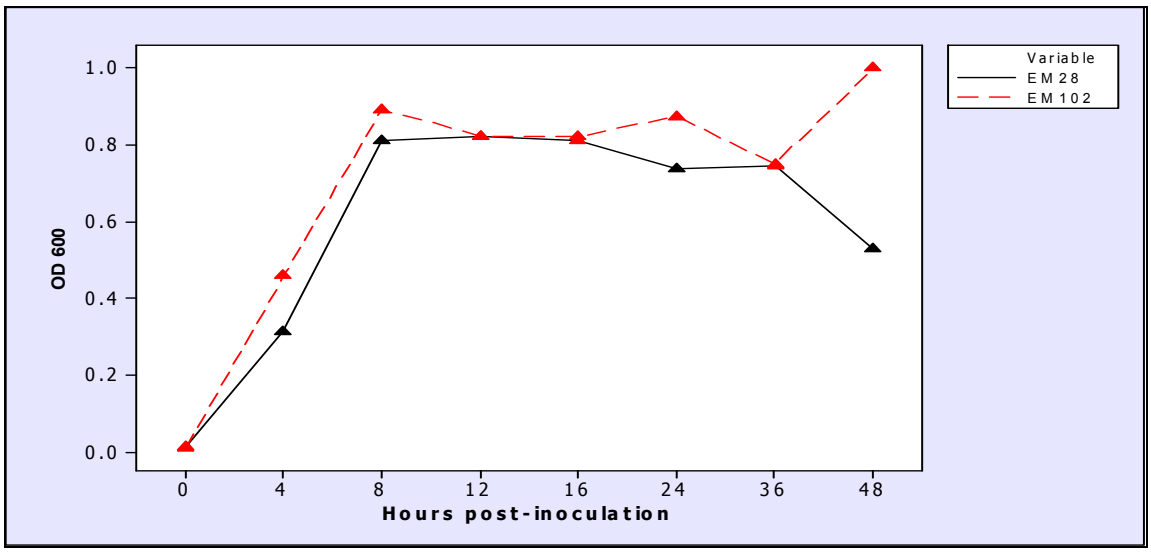


Figure 6.11. Growth curves for EM28 and EM102 expressed as OD_{600} values. Strains were grown up in PPY medium as described in section 2.5.2.

Figure 6.12 shows toxin production by the two strains over a 48 hour period.

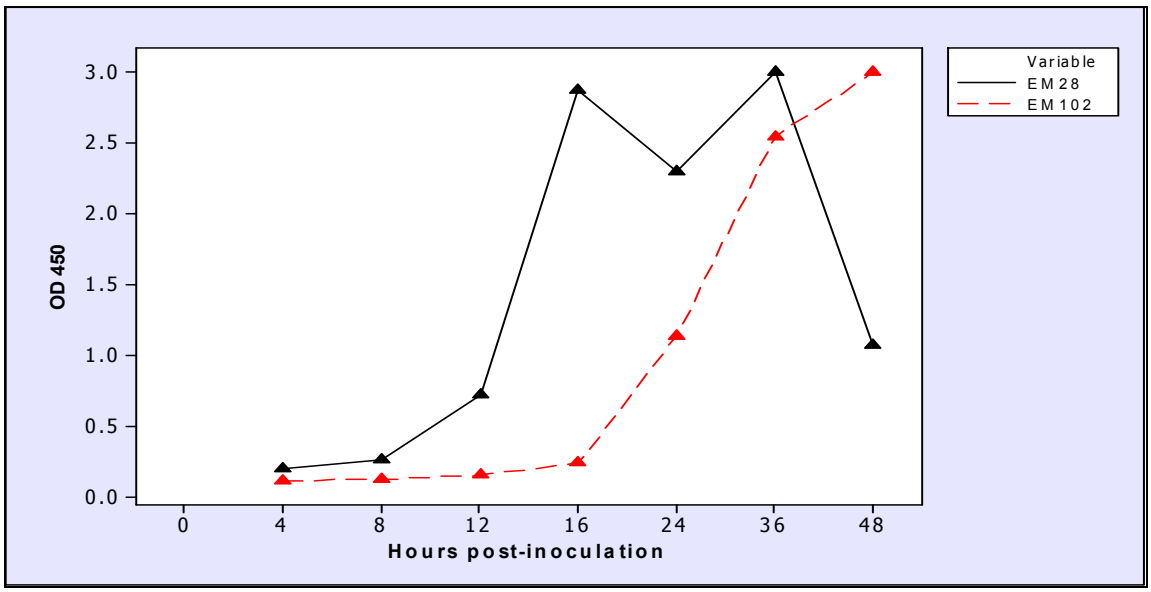
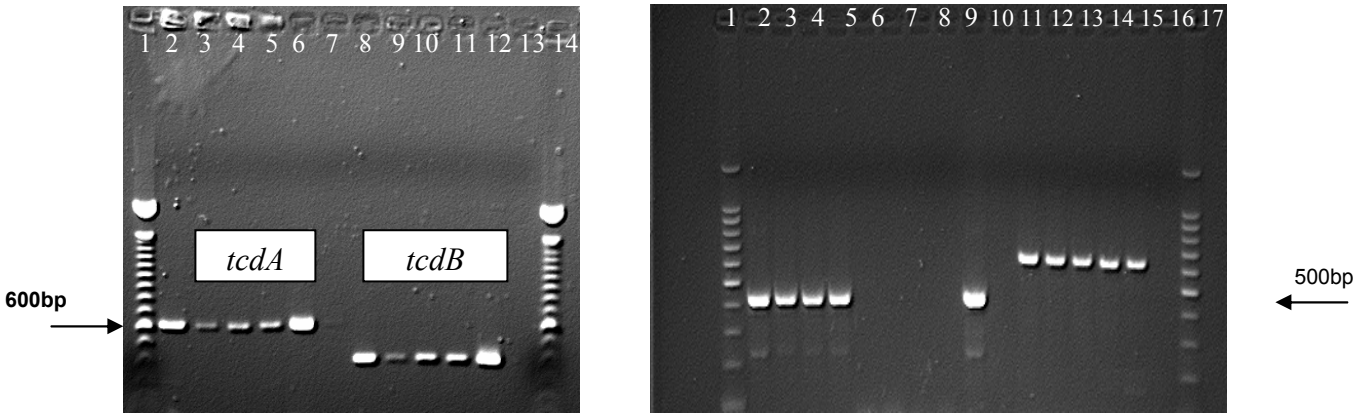


Figure 6.12. Toxin production by EM28 and EM102 as measured by Toxin A + B ELISA (TechLab) and expressed as OD_{450} values. A reading of 3·0 is the maximum value for the toxin assay.

It can be seen that the two strains differ in their pattern of toxin production. EM28 starts producing toxins after 8 hours and the level is close to its peak by 16 hours. In comparison, EM102 starts producing toxins after 16 hours and reaches an equivalent level to EM28 after 48 hours. The apparent fall in the level of toxins produced by EM28 at 24 hours is likely to be an experimental anomaly or reflects breakdown of the toxins or their complexing with other molecules in the growth medium. The greatest difference in toxin production is seen at 16 hours post-inoculation, during early/mid stationary phase, and therefore this time point was chosen as the focus of further analysis.

Expression of *tcdA* and *tcdB*

Initial experiments using EM28 and EM102 indicated that expression of both *tcdA* and *tcdB* was higher at 16 hours in EM28. By 24 hours post-inoculation (late stationary phase) expression in this strain had reduced and was comparable to that seen in EM102 (Figure 6.13).

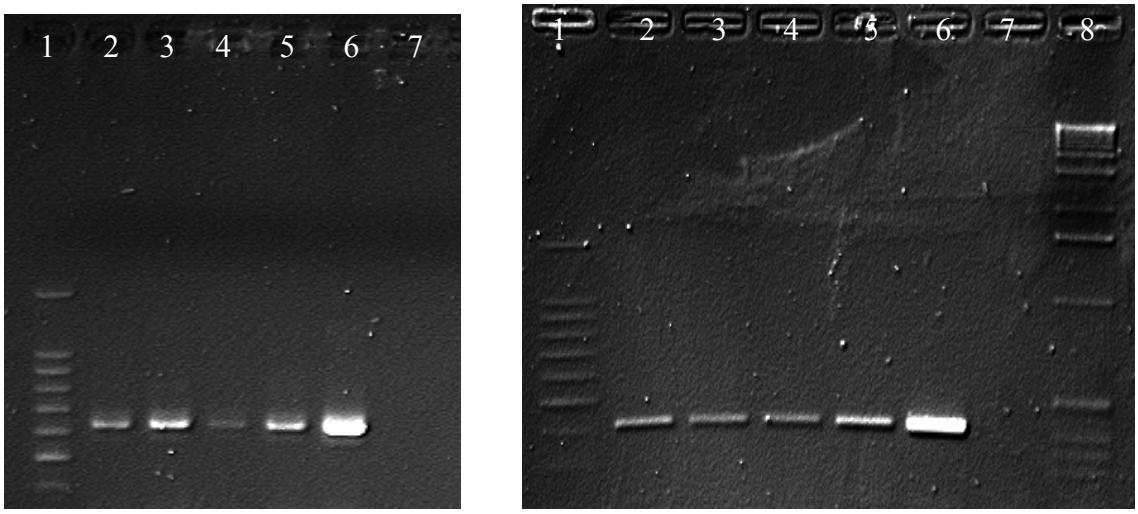


(a) **(b)**

Figure 6.13. RT-PCR amplification of *tcdA* and *tcdB* fragments. **(a).** Lanes 1 and 14 100bp ladder (Invitrogen), **lanes 2-7 *tcdA* (624bp):** lane 2 EM28 at 16 hours, lane 3 EM102 at 16 hours, lane 4 EM28 at 24 hours, lane 5 EM102 at 24 hours, lane 6 630 gDNA, lane 7 negative control. **Lanes 8-13 *tcdB* (412bp):** lane 8 EM28 at 16 hours, lane 9 EM102 at 16 hours, lane 10 EM28 at 24 hours, lane 11 EM102 at 24 hours, lane 12 630 gDNA, lane 13 negative control.

(b). Lanes 2 and 18 100bp ladder (Promega). **Lanes 2-11 *tcdB* (412bp):** lane 2 EM28 at 16 hours, lane 3 EM102 at 16 hours, lane 4 EM28 at 24 hours, lane 5 EM102 at 24 hours, lanes 6-9 RNA preps, lane 10 630 gDNA, lane 11 negative control. **Lanes 12-17 *tcdA* (624bp):** lane 12 EM28 at 16 hours, lane 13 EM102 at 16 hours, lane 14 EM28 at 24 hours, lane 15 EM102 at 24 hours, lane 16 630 gDNA, lane 17 negative control.

These findings were not replicated in subsequent experiments. Figure 6.14 reveals a reversed position with EM28 showing a lower level of expression of both genes at both time points. When *tcdA* and *tcdB* expression in EM47 (intermediate resistance), EM96 (sensitive) and EM125 (resistant) was examined, greater levels of expression were seen in the intermediate and sensitive strains at 16 hours post-inoculation (Figure 6.15).



(a) (b)

Figure 6.14. (a). RT-PCR amplification of *tcdA*. Lane 1 100bp ladder (Promega). Lane 2 EM28 at 16 hours, lane 3 EM102 at 16 hours, lane 4 EM28 at 24 hours, lane 5 EM102 at 24 hours, lane 6 630 gDNA, lane 7 negative control.

(b). RT-PCR amplification of *tcdB*. Lanes 1 and 8 100bp ladder (Promega). Lane 2 EM28 at 16 hours, lane 3 EM102 at 16 hours, lane 4 EM28 at 24 hours, lane 5 EM102 at 24 hours, lane 6 630 gDNA, lane 7 negative control.

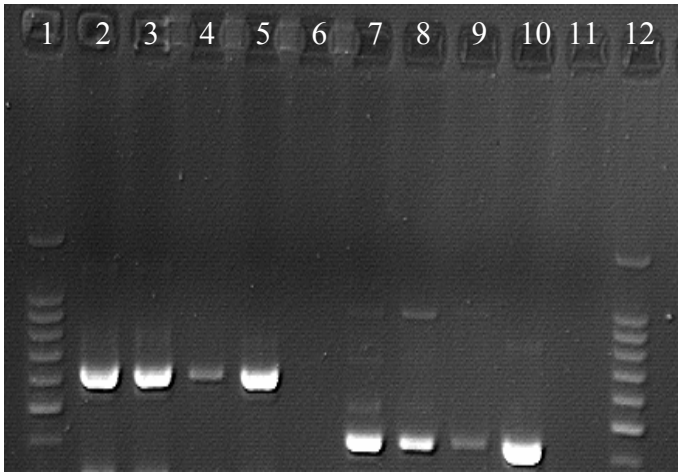


Figure 6.15. RT-PCR amplification of *tcdA* and *tcdB* fragments. Lanes 1 and 12 100bp ladder (Promega). **Lanes 2-6 *tcdA* (624bp):** lane 2 EM47, lane 3 EM96, lane 4 EM125, lane 5 630 gDNA, lane 6 negative control. **Lanes 7-11 *tcdB* (412bp):** lane 7 EM47, lane 8 EM96, lane 9 EM125, lane 10 630 gDNA, lane 11 negative control.

A final experiment in which three sensitive strains (EM96, EM102 & EM120) and three resistant strains (EM28, EM125 & EM184) were cultured simultaneously demonstrated no obvious trend in expression between the two groups of strains at 16 hours post-inoculation (Figure 6.16).

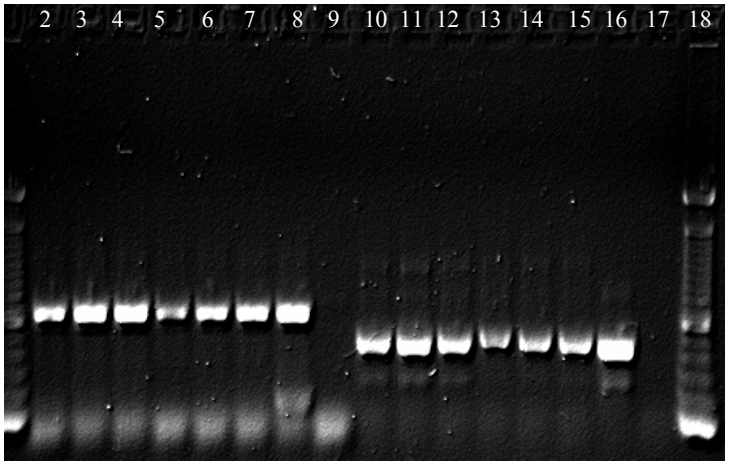


Figure 6.16. RT-PCR amplification of *tcdA* and *tcdB* fragments. Lanes 1 and 18 100bp ladder (Invitrogen). **Lanes 2-9 *tcdA* (624bp):** lane 2 EM28, lane 3 EM96, lane 4 EM102, lane 5 EM120, lane 6 EM125, lane 7 EM184, lane 8 630 gDNA, lane 9 negative control. **Lanes 10-17 *tcdB* (412bp):** lane 10 EM28, lane 11 EM96, lane 12 EM102, lane 13 EM120, lane 14 EM125, lane 15 EM184, lane 16 630 gDNA, lane 17 negative control.

Taken as a whole these experiments indicate that there is no reproducible difference in the expression of *tcdA* and *tcdB* between resistant and sensitive strains in early/mid stationary phase.

Measurement of toxin production by ELISA

Two separate experiments were performed. All 28 strains for which the *gyrA* and *gyrB* genes had been sequenced were grown up in PPY with the growth medium sampled 16 hours post-inoculation and toxin concentration determined by ELISA, as described in section 2.5.3. The mean of the two readings was taken and toxin production by the two groups of strains was compared by means of a Mann-Whitney test. The data are shown in Figure 6.17. Sensitive strains have a greater interquartile range (1.46 vs. 1.08), however, median values are little different between sensitive and resistant strains (0.29 and 0.45 respectively; p=0.8).

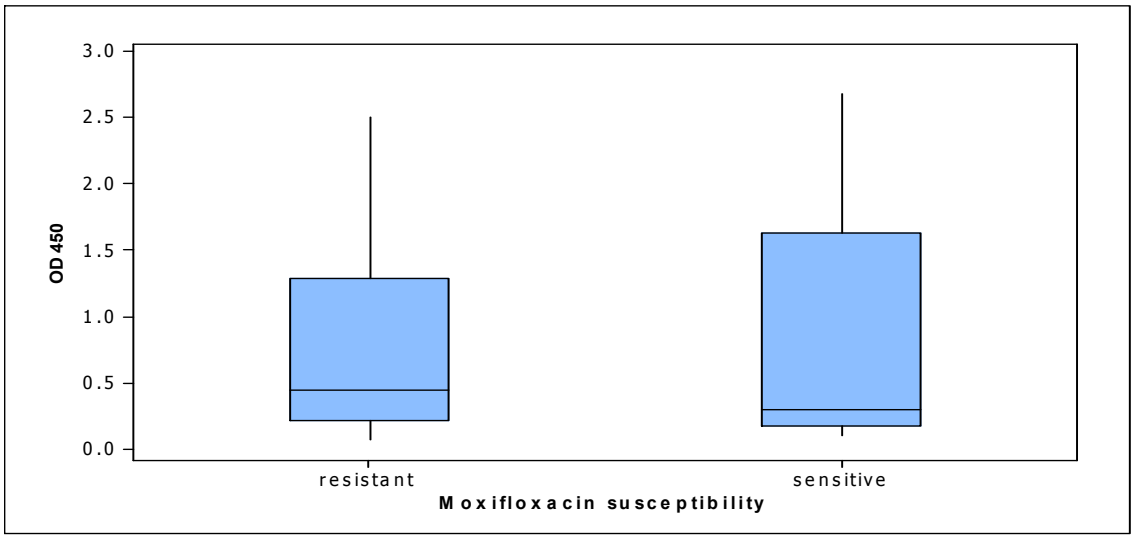


Figure 6.17. Comparison of toxin production 16 hours post-inoculation by resistant (n=14) and sensitive strains (n=14) as measured by ELISA and expressed as OD_{450} values. Boxes represent the range between quartile 1 and quartile 3, with the median value represented by the horizontal line. The whiskers indicate highest and lowest values.

If the data are analysed excluding those strains for which no *tcdC* sequence could be obtained the picture is little different (Figure 6.18). Sensitive strains now have a smaller interquartile range than resistant (0.77 vs. 1.08) but the median value is reduced only marginally to 0.26. There remains no statistically significant difference between the two groups (p=0.7).

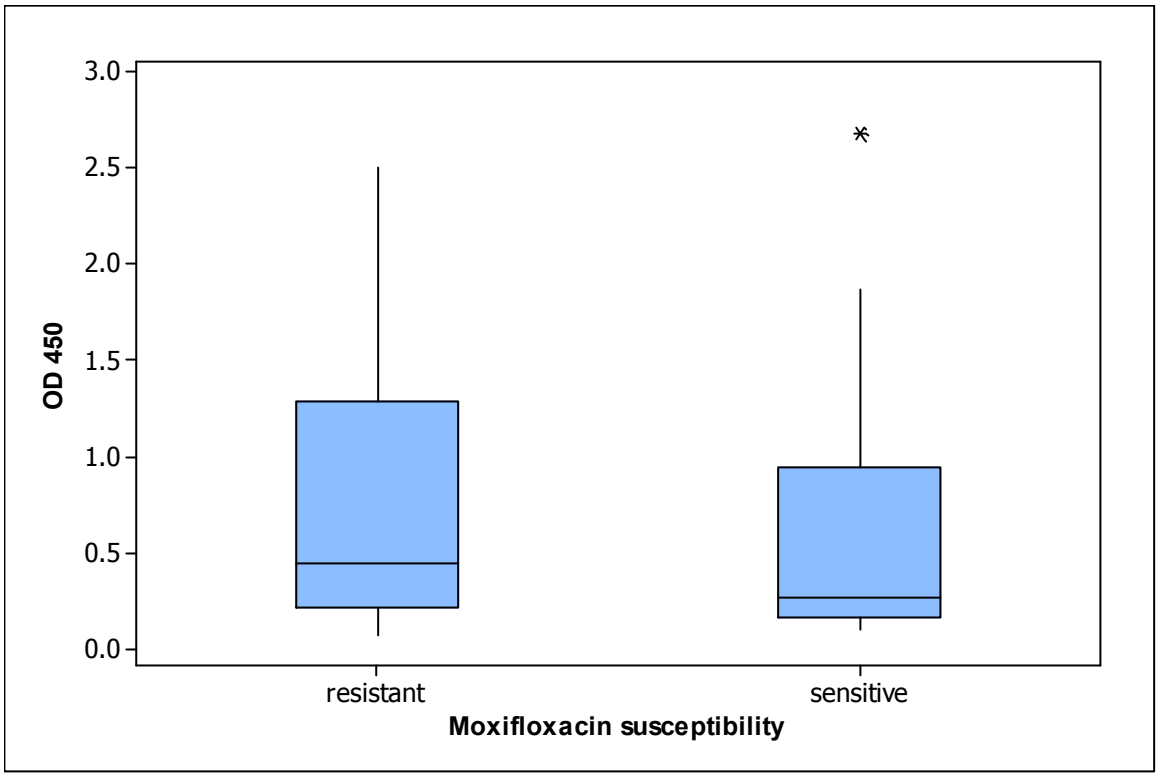


Figure 6.18. Comparison of toxin production 16 hours post-inoculation by resistant (n=14) and sensitive strains (n=10, excluding those without sequenced *tcdC* fragment) as measured by ELISA and expressed as OD_{450} values. Boxes represent the range between quartile 1 and quartile 3, with the median value represented by the horizontal line. The whiskers indicate highest and lowest values, with outliers indicated by an asterisk.

Correlation between transcription and toxin production as measured by ELISA

The data obtained in this study are insufficient to determine whether a correlation exists in the strains under examination between transcription of *tcdA* and *tcdB* and toxin production as measured by ELISA. Levels of transcription were ascertained at one, or in some instances two, time points only. The concentration of toxin present in the growth medium at those time points, as measured by ELISA, does not reflect transcription at that point alone; the growth culture is a closed system and toxin concentration therefore reflects transcription, translation and secretion from inoculation to the point of sampling.

6.4.4 Growth comparisons

The growth of four resistant and four sensitive strains, as measured by OD, was recorded over a 24 hour period (Figure 6.19). With the exception of EM83 (sensitive) there is little discernable difference in growth between the sensitive and resistant strains.

The mean OD values for the two groups of strains at each time point are shown in Figure 6.20. The data suggest that growth occurs more rapidly and to a higher level in sensitive strains, however, the difference is not significant (p=0.2) and may be skewed by the presence of EM83.

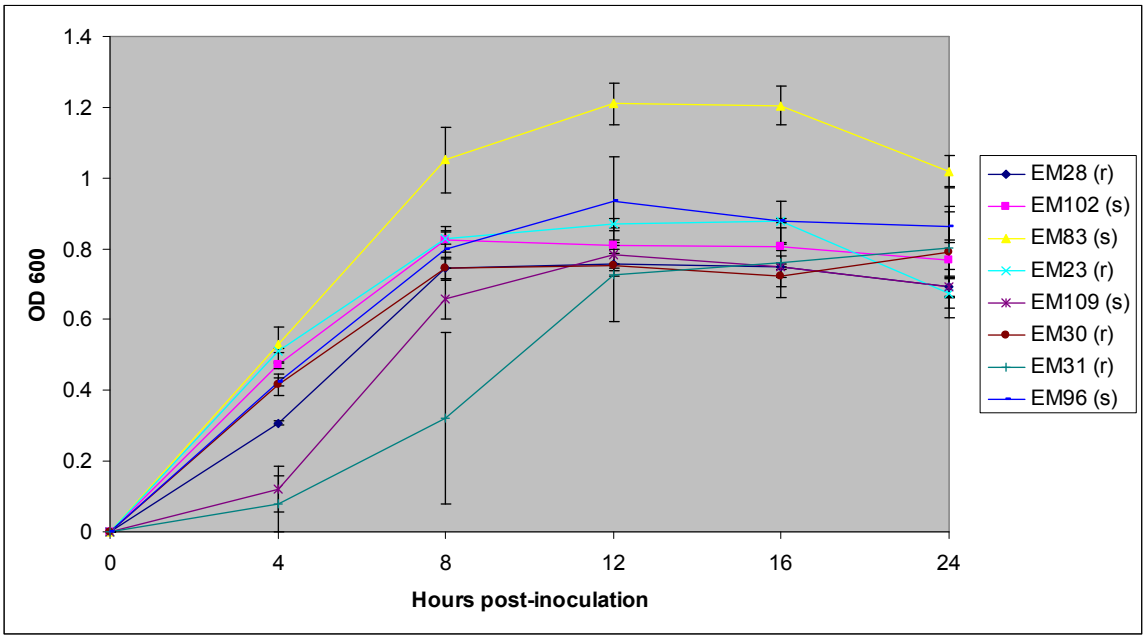


Figure 6.19. Growth expressed as OD_{600} values over a 24 hour period. (R = resistant, S = sensitive.). Each data point represents the mean of three separate experiments. Error bars represent the standard error of the mean.

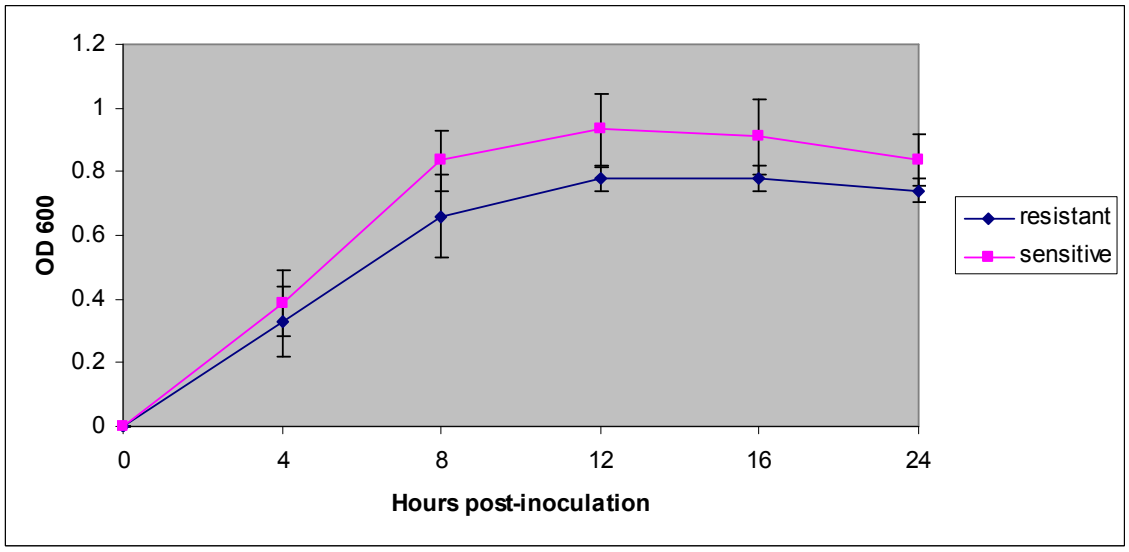


Figure 6.20. Growth expressed as averaged OD_{600} values for resistant and sensitive strains. Error bars represent the standard error of the mean.

A further study comparing the growth of EM96-R and its parent EM96 over a 24 hour period showed that the mutant grew more rapidly and achieved higher OD values than did the parent, indicating that the fitness of the mutant was not impaired by its reduced susceptibility to moxifloxacin (Figure 6.21).

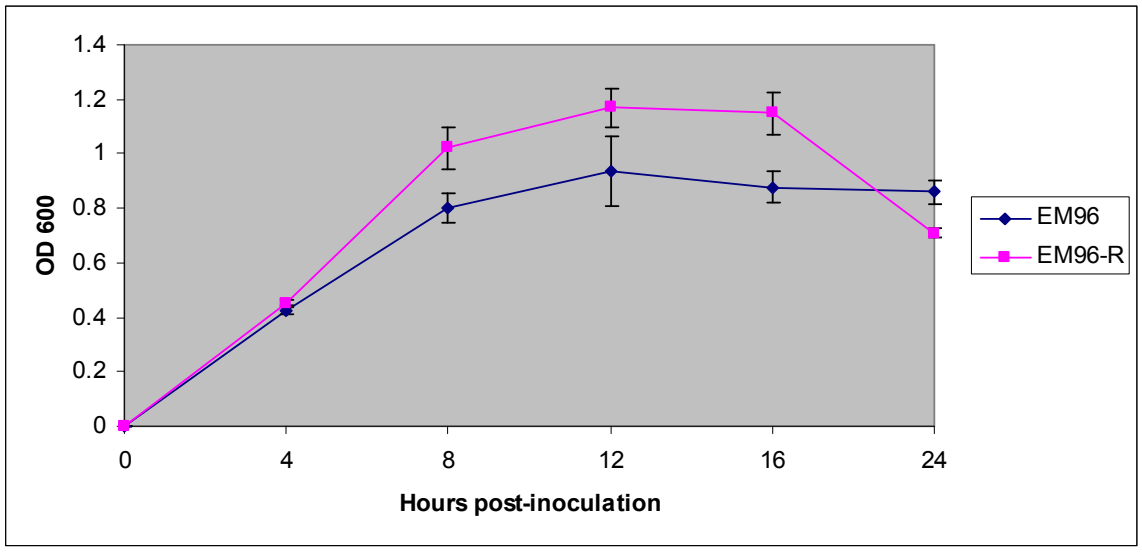


Figure 6.21. Comparative growth of EM96-R and its parent EM96, expressed as OD_{600} values. Each data point represents the mean of three separate experiments. Error bars represent the standard error of the mean.

6.5 Discussion

6.5.1 Mechanisms of moxifloxacin resistance

As noted in the introduction to this chapter, fluoroquinolone resistant strains of *C. difficile* characterised to date have shown amino acid changes in the quinolone resistance determining region of GyrA and GyrB. GyrB mutations are less common than GyrA and tend to confer low levels of resistance (Spigaglia *et al.,* 2008). The GyrA amino acid substitution Thr82→ Ile has been reported by a number of groups (Ackermann *et al.,*

2001; Dridi *et al.,* 2002; Drudy *et al.,* 2007; Schmidt *et al.,* 2007; Spigaglia *et al.,* 2008). This mutation has been demonstrated in all ribotype 027 strains characterised so far (Drudy *et al.,* 2007; Spigaglia *et al.,* 2008). Thr82 corresponds to Ser83 in *Escherichia coli,* the mutation of which alters the structure of the quinolone binding site near the interface of the enzyme and DNA (Hooper, 1999). Drudy *et al.,* (2006) reported the presence of the GyrB substitution Asp426→Val in a cluster of 5 clonal toxin A-negative, toxin B-positive outbreak strains found to be resistant to five fluoroquinolones. Asp426 is thought to be a key site in GyrB; mutations in the corresponding codon in *E. coli, Staphylococcus aureus* and *Streptococcus pneumoniae* are associated with fluoroquinolone resistance (Hooper, 1999). The presence of the GyrA amino acid substitution Thr82→ Ile was identified in all the resistant strains examined, with the exception of a strain with intermediate resistance and EM96-R. Neither of these strains carried a mutation in *gyrB*, suggesting that reduced susceptibility to moxifloxacin in these strains is mediated by a different mechanism. Efflux pumps are known to be present in many bacteria and can confer resistance to a variety of molecules, including antibiotics, by extrusion (Lebel *et al.,* 2004). It was hypothesised that the *cme* and *cdeA* genes, both encoding a putative efflux pump, may play a role in reduced susceptibility to moxifloxacin; however, the data presented here from experiments examining the expression of both genes, do not support this hypothesis. In the case of *cdeA* overexpression of the gene was seen in one, resistant, strain only. While early experiments suggested that overexpression of *cme* was seen only in those strains exhibiting reduced susceptibility to moxifloxacin, these findings were not reproduced in subsequent experiments.

This study is believed to be the first to examine CD3197, a transcriptional regulator located upstream of *cme*. Three novel mutations in the putative promoter region of CD3197, in the form of tandem forward repeat insertions or deletions, were identified. The repeat sequence appears to be unique to *C. difficile*. Repeat sequences are widely found in bacterial genomes and those found within a gene's regulatory region can function as a transcriptional on/off switch (van der Woude & Baumler, 2004). It was hypothesised that the presence of these mutations would be associated with overexpression of *cme*; however, the data refute this hypothesis, with only one strain with a variant genotype being found to overexpress this gene. This may be a reflection of the position of the repeats, which lie 25bp downstream of the -10 promoter element and 145bp upstream of the CD3197 gene start site. Repeat sequences affecting transcription levels are more commonly found between the -35 and -10 promoter elements (Moxon *et al.,* 1994), where they influence the binding efficiency of RNA polymerase.

No association was established between the presence of these mutations and resistance or sensitivity to moxifloxacin, with the variant genotypes being present in both sensitive and resistant organisms. The conservation of a 19bp deletion in EM96-R further suggests that selection in the presence of moxifloxacin does not act in this region of the genome.

6.5.2 Moxifloxacin resistance and fitness

Studies examining virulence factors and antimicrobial susceptibility in bacterial strains have produced conflicting findings. Resistance to quinolones and fluoroquinolones among uropathogenic *E. coli* isolates has been shown to be associated with reductions in virulence traits and intrinsically lower virulence strains (Moreno *et al.,* 2006; Horcajada *et al.,* 2005). Strains of *Pseudomonas aeruginosa* overexpressing a type 1 beta-lactamase were found to be less virulent in a mouse model of acute pneumonia (Ramisse *et al.,* 2000). On the other hand, Godoy *et al.,* (2003) found no significant association between pathogenicity and antimicrobial susceptibility or resistance among *Helicobacter pylori* clinical isolates. Furthermore, Billstrom *et al.,* (2008) found a significant correlation between the presence of the gene encoding the enterococcal surface protein, a putative virulence factor, and resistance to ampicillin, ciprofloxacin and imipenem in clinical isolates of *Enterococcus faecium.* It is believed that the study presented here is the first to examine the relationship between fitness, as measured by growth and the production of the virulence factors toxins A and B, and susceptibility or resistance to moxifloxacin. This study found no correlation between these features, although sensitive strains did have a greater range of toxin levels and a higher upper value than the resistant strains. However, median values were little different. These results indicate that the presence of the moxifloxacin resistance mechanism(s) do not impose a fitness cost.

In conclusion, this study shows the primary mechanism of resistance to moxifloxacin among this population of clinical strains to be the presence of a *gyrA* mutation that results in an amino acid substitution at position 82. However, the absence of this

mutation or any mutation in *gyrB* in two strains of reduced susceptibility suggests that fluoroquinolone resistance may be mediated in part by another mechanism. The data suggest that the putative efflux pumps encoded by *cme* and *cdeA* do not play a role in the strains examined.

No association between virulence, as measured by growth and toxin production, and moxifloxacin resistance or susceptibility was demonstrated.

7. Conclusions

This chapter summarises the conclusions drawn from the three studies comprising this thesis. Where appropriate suggestions are made as to how these findings could usefully be expanded upon.

To reiterate the aims and hypotheses of the three studies:

1. To enumerate immune cell populations within the lamina propria of colonic tissue from cases, carriers and controls. It was hypothesised that cases would have fewer immune cells than both carriers and controls, while immune cell populations of carriers would approximate those of controls.

2. To determine the prevalence of specific host single nucleotide polymorphisms (SNPs) in the *TLR2, TLR5* and *IL-8* genes in the study population and to assess whether their presence was associated with susceptibility to CDI.

3. To determine the mechanisms of moxifloxacin resistance in a collection of clinical isolates.

4. To determine whether the competitive advantage conferred by resistance to moxifloxacin influences the fitness of *C. difficile* isolates, in particular growth and the expression of the virulence factors toxins A and B.

Carriers were found to have fewer of all four cell types quantified than both cases and controls, while cases had fewer of all cell types than controls. Multivariate analysis of total cell counts, however, indicated that no statistically significant difference existed between the study groups. A larger, prospective study in which a series of faecal and tissue specimens were taken prior to and during hospitalisation would be required to cast light on the role of the mucosal immune response in determining the outcome of infection.

Despite the lower median cell counts seen in carriers the immune response mounted by these individuals is protective against the development of symptomatic colonisation. To understand the nature of this protective response it would be necessary to characterise the B and plasma cells in terms of the immunoglobulin class secreted and the antigen-specificity of the antibodies produced. Characterisation of the antigen-specificity of effector $CD4^+$ T cells, particularly the T_H2 subtype, would also be required. Immunohistochemical staining of tissue samples for immunoglobulin class, particularly IgA, is difficult to interpret. Treatment of tissue samples to release individual cells and subsequent analysis of these cells by a technique such as FACS (fluorescence-activated cell sorting) would be more effective. Antigen-specificity could be determined using a technique such as ELISPOT (enzyme-linked immunosorbent spot).

The data are suggestive of an association between the presence of host SNPs and increased susceptibility to CDI. The variant IL-8 and TLR2 genotypes were carried by cases and carriers while the variant TLR5 genotype was carried by cases only. No variant genotypes were present in control subjects. Of the three individuals carrying the

IL-8 variant genotype associated with an increased susceptibility to CDI, one case with this genotype was heterozygous for both the TLR2 and TLR5 variant alleles. The two carriers with this genotype were both homozygous for the wild-type TLR2 and TLR5 alleles.

Published data have demonstrated an association between the variant IL-8 -251 A/A genotype and increased susceptibility to CDI when present in conjunction with a further immune response defect. This study lends support to those findings with the presence of the $TLR5^{392STOP}$ polymorphism representing such a defect. The data are also suggestive of an association between the TLR5 polymorphism and CDI when present in isolation. The TLR2 variant allele was found only in one case and one carrier, suggesting a possible association with infection. The data suggest that a larger study examining the presence of the IL-8, TLR2 and TLR5 polymorphisms would be of merit. Analysis of the SNPs in conjunction with other aspects of the host immune response, such as serum antibody levels and mucosal immune cell populations, could prove useful in identifying combinations of host immune factors influencing susceptibility to CDI. Of particular interest would be a study seeking to determine whether an association existed between the presence of the TLR SNPs and the character of the immune cell populations of the colonic lamina propria. Immunoglobulin class switching by B and plasma cells is mediated by the production of cytokines by cells of the innate immune system. If TLR-mediated signalling upon recognition of *C. difficile* by these cells is altered by the presence of polymorphisms it may be reflected in the balance of IgG and IgA-secreting cells in the lamina propria.

Moxifloxacin resistant isolates carry a mutation in *gyrA*. All resistant isolates tested, with the exception of an isolate with intermediate resistance and a moxifloxacin mutant with reduced susceptibility, carried the common *gyrA* mutation ACT→ATT (Thr82→Ile). This mutation was not present in any sensitive isolates. Neither the intermediate isolate nor the moxifloxacin mutant carried any other *gyrA* mutation. No mutations in *gyrB* were found in any isolate. Semi-quantitative PCR analysis of expression of the putative efflux pumps *cme* and *cdeA* found no correlation between overexpression and moxifloxacin resistance, suggesting that these genes do not play a role in moxifloxacin resistance. Three novel mutations in the putative promoter region of CD3197, a MerR family transcriptional regulator found immediately upstream of *cme*, were identified. No association between the presence of these mutations and overexpression of *cme* or resistance or sensitivity to moxifloxacin was found.

Although the data derived from semi-quantitative PCR did not ultimately support the hypothesis of a role for *cme* in moxifloxacin resistance, preliminary work on its expression strongly suggested that overexpression was seen in resistant isolates. There would be merit in looking further at this using the more sensitive and discriminatory technique of qPCR, possibly at a greater number of time points.

The 19bp repeat sequence found in the putative promoter region of CD3197 appears to be unique to *C. difficile*. The data reported in this study suggest that variation in the number of repeat sequences is not associated with variability in transcription of *cme*. However, transcription is not the only process that may be affected by the presence of repeat sequences. Phenotypic variation in a number of bacterial species has been shown to be governed by variations in repeat sequence numbers that act as translational

switches (Moxon *et al.,* 1994). This study did not look at translational differences between isolates and an analysis of cellular levels of the CD3197 protein could shed light on two questions. Firstly, do variations in the number of 19bp tandem repeats influence translation of these transcripts and secondly, does the relationship between levels of CD3197 and expression of *cme* indicate that CD3197 is indeed a transcriptional regulator of *cme*.

There is no difference in the fitness, as measured in terms of growth and toxin production, of moxifloxacin resistant and moxifloxacin sensitive isolates. A comparison of the growth of resistant and sensitive isolates over a 24 hour period indicated that growth occurred more rapidly and to a higher level in sensitive isolates, however, the difference was not significant. A further study comparing the growth of a moxifloxacin mutant with reduced susceptibility and its sensitive parent isolate over a similar period showed that the mutant grew more rapidly and achieved higher OD values than did the parent, indicating that the fitness of the mutant was not impaired by its reduced susceptibility to moxifloxacin.

A series of experiments examining toxin gene expression by means of semi-quantitative PCR demonstrated that there was no reproducible difference in the expression of *tcdA* and *tcdB* between resistant and sensitive isolates in late exponential/early stationary phase. Examination of toxin A and B production by ELISA showed that sensitive isolates have a greater interquartile range of OD values, however, median values were little different between sensitive and resistant isolates.

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Appendix 1. Primer sequences and sources

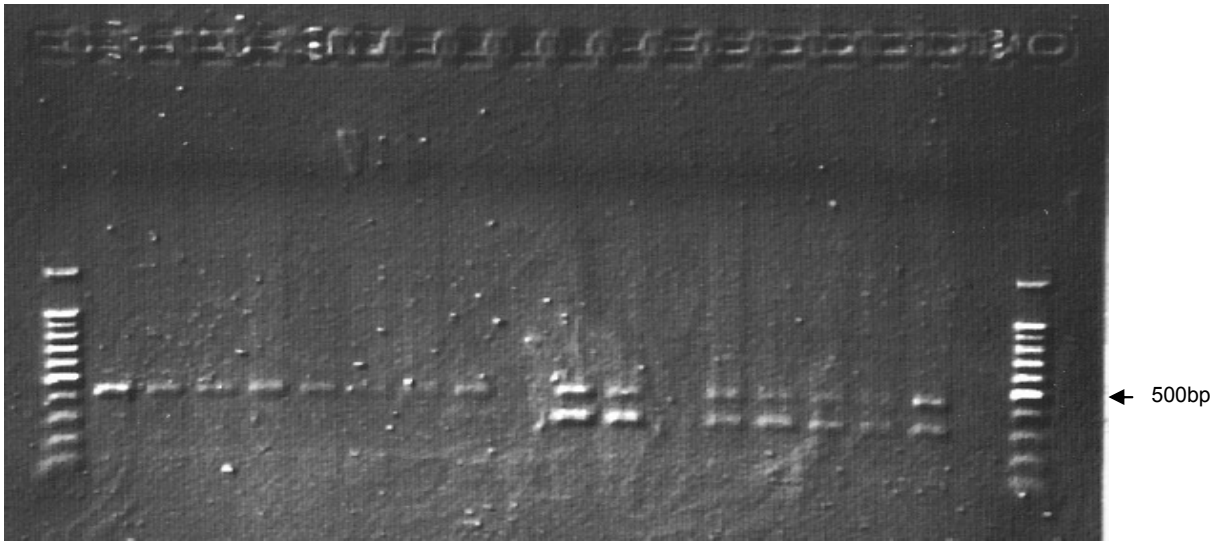
Gene	Primer	Sequence	Source
16s rRNA	F-968-GC	5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGG GCACGGGGGGAACGCGAAGAACCTTAC-3'	Nübel et al., (1996)
	R-1346	5'-TAGCGATTCCGACTTCA-3'	
cdd-3	Tim 6	5'-TCC AAT ATA ATA AAT TAG CAT TCC A-3'	Cohen et al., (2000)
	Struppi 6	5'-GGC TAT TAC ACG TAA TCC AGA TA-3'	
cdtA	F	5'-TGAACCTGGAAAAGGTGATG-3′	Stubbs et al., (2000)
	R	5′-AGGATTATTTACTGGACCATTTG-3′	
cdtB	F	5′-CTTAATGCAAGTAAATACTGAG-3′	Stubbs et al., (2000)
	R	5′-AACGGATCTCTTGCTTCAGTC-3′	
cme	F	5'-GCTGGTGTATGGGCAGACTA-3'	Designed with Primer 3 from published sequence of strain 630.
	R	5'-CATTGCGTTTACAGCAGGTG-3'	
gyrA	GyrAF	5'-TTG AAA TAG CGG AAG AAA TGA-3'	Drudy et al., (2006).
	GyrAR	5'-TTG CAG CTG TAG GGA AAT C-3'	
gyrB	GyrBF	5'-GAA GGT CAA ACT AAA ACA AA-3'	Drudy et al., (2006).
	GyrBR	5'-GGG CTC CAT CTA CAT CAG-3'	
IL-8	con	5'-TGC CCC TTC ACT CTG TTA AC-3'	Hull et al., (2000)
	251T	5'-CCA CAA TTT GGT GAA TTA TCA AA-3'	
	251A	5'-CCA CAA TTT GGT GAA TTA TCA AT-3'	
	HLA-DRB1 F	5'-TGCCAAGTGGAGCACCCAA-3'	Internal control primer
	HLA-DRB1R	5'- GCATCTTGCTCTGTGCAGAT-3'	Internal control primer
Ribotyping	F	5'-CTG GGG TGA AGT CGT AAC AAG G-3'	O'Neill et al., (1996)
	R	5'-GCG CCC TTT GTA GCT TGA CC-3'	
tcdA expression	TA1	5'-ATG ATA AGG CAA CTT CAG TGG-3'	Spigaglia & Mastrantonio (2002).
	TA2	5'-TAA GTT CCT CCT GCT CCA TCA A-3'	
tcdB expression	TB1	5'-GAG CTG CTT CAA TTG GAG AGA-3'	Spigaglia & Mastrantonio (2002).
	TB2	5'-GTA ACC TAC TTT CAT AAC ACC AG-3'	
tcdA toxinotyping	A3C	TAT TGA TAG CAC CTG ATT TAT ATA CAA G	Rupnik et al., (1997, 1998)
	A4N	5'-TTA TCA AAC ATA TAT TTT AGC CAT ATA TC-3'	

Gene	Primer	Sequence	Source
tcdB toxinotyping	B1C	5’-AGA AAA TTT TAT GAG TTT AGT TAA TAG AAA-3’	Rupnik et al., (1997, 1998)
	B2N	5’-CAG ATA ATG TAG GAA GTA AGT CTA TAG-3’	
tcdC	F	5’-GAGCCTTGTAACTGTTTATTTGC-3’	Designed with Primer 3 from published sequence of reference strain VPI10463
	R	5’-TTTGTAAAATTATGCTTAGGGGAAA-3’	
TLR2	F	5’-TAT GGT CCA GGA GCT GGA GA-3’	Ogus et al., (2004)
	R	5’-TGA CAT AAA GAT CCC AAC TAG ACA A-3’	
	G	5’-GGTCTTGGTGTTCATTATCTTCC-3’	
	A	5’-GGTCTTGGTGTTCATTATCTTCT-3’	
TLR5	F T5-11	5' - GGTAGCCTACATTGATTTGC-3'	Hawn et al., (2003)
	R T5-23	5' - GAGAATCTGGAGATGAGGTACCCG - 3'	
MerR	F	5’-CACGACATCAGAGGTAGCAGA-3’	Designed with Primer 3 from published sequence of reference strain 630
	R	5’-CAAGTGATGAGCCAAATAAGG-3'	
CD3197	F	5'-CGGTTATCTGTGGTGGCTATG-3'	Designed with Primer 3 from published sequence of reference strain 630
	R	5'-AGTGCTTCCTCGGCATTAGA-3'	
cdeA	F	5'-TCACTGTTTTCAGCACCAAAA-3'	Dridi *et al.,* (2004)
	R	5'-TTGCTTGGTGCAACAGATAAG-3'	

Appendix 2. Representative gels from the genetic polymorphisms study reported in Chapter 5

TLR2

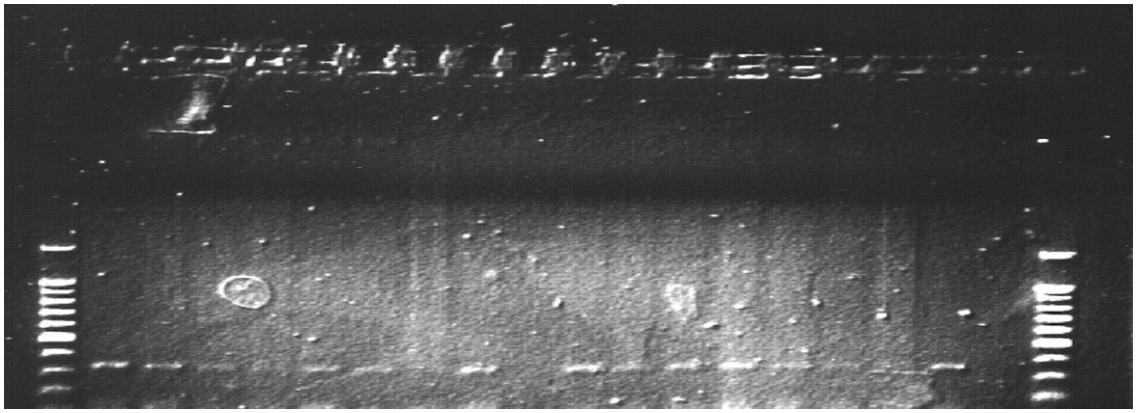
In this duplex reaction the internal control band is 470bp in length while the allele-specific band is 328bp long.



Lanes 1 and 20 100bp ladder; lanes 2 – 10 A allele and lanes 11-19 G allele, lanes 2 and 11 AW6; lanes 3 and 12 AW31; lanes 4 and 13 AW75; lanes 5 and 14 AW81; lanes 6 and 15 AW31; lanes 7 and 16 AW82, lanes 8 and 17 AW88; lanes 9 and 18 AW81; lanes 10 and 19 negative controls. Where identification numbers are duplicated DNA has been extracted from two different tissue samples from that individual.

IL-8

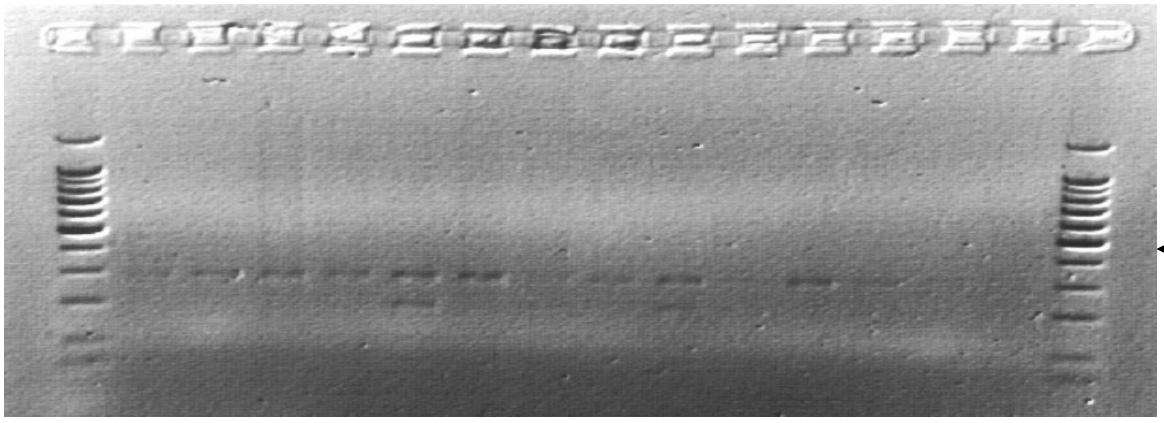
The allele-specific band is 336bp in length. Primers were obtained for use in a duplex reaction to give an internal control band of 796bp. However, bands were not always seen for this reaction when allele-specific bands were present and non-specific bands were also observed. For these reasons certain PCR reactions were run without the control primers, as illustrated in the gel below.



Lanes 1 and 20 100bp ladder; lanes 2 – 10 A allele and lanes 11-19 G allele, lanes 2 and 11 AW6; lanes 3 and 12 AW31; lanes 4 and 13 AW75; lanes 5 and 14 AW81; lanes 6 and 15 AW31; lanes 7 and 16 AW82, lanes 8 and 17 AW88; lanes 9 and 18 AW81; lanes 10 and 19 negative controls. Where identification numbers are duplicated DNA has been extracted from two different tissue samples from that individual.

TLR5

The PCR product is 276bp in length. Variant alleles are identified by restriction digest using *Dde*I which cuts the variant allele only; the products of such a restriction have been run on the gel shown below. Cut products can be seen in lanes 6 and 10.



Lanes 1 and 16 100bp ladder; lane 2 AW57; lane 3 AW6; lane 4 AW7; lane 5 AW96; lane 6 AW97; lane 7 AW31; lane 8 AW81; lane 9 AW31; lane 10 AW95; lane 11 AW81; lane 12 AW21; lane 13 AW107; lane 14 AW108; lane 15 AW95. Where identification numbers are duplicated DNA has been extracted from two different tissue samples from that individual.

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Molecular characterization and antimicrobial susceptibility patterns of *Clostridium difficile* strains isolated from hospitals in south-east Scotland

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Clostridium difficile isolates (n=149) collected in south-east Scotland between August and October 2005 were typed by four different methods and their susceptibility to seven different antibiotics was determined. The aims were to define the types of strain occurring in this region and to determine whether there were any clonal relationships among them with respect to genotype and antibiotic resistance pattern. Ribotyping revealed that 001 was the most common type (n=113, 75.8 %), followed by ribotype 106 (12 isolates, 8.1 %). The majority of the isolates (96.6 %, n=144) were of toxinotype 0, with two toxinotype V isolates and single isolates of toxinotypes I, IV and XIII. PCR and restriction analysis of the *fliC* gene from 147 isolates gave two restriction patterns: 145 of pattern VII and two of pattern I. Binary toxin genes were detected in only three isolates: two isolates of ribotype 126, toxinotype V, and one isolate of ribotype 023, toxinotype IV. S-types showed more variation, with 64.5 % (n=40) of the common S-type (4939) and 21 % (n=13) of S-type 4741, with six other S-types (one to three isolates each). All ribotype 001 isolates were of the same S-type (4939), with three isolates of other ribotypes being this S-type. No resistance was found to metronidazole or vancomycin, with resistance to tetracycline only found in 4.3 % of the isolates. A high proportion of isolates were resistant to clindamycin (62.9 %), moxifloxacin, ceftriaxone (both 87.1 %) and erythromycin (94.8 %). Resistance to three antibiotics (erythromycin, clindamycin and ceftriaxone) was seen in 66 isolates, with erythromycin, ceftriaxone and moxifloxacin resistance seen in 96 isolates. Resistance to all four of these antibiotics was found in 62 isolates and resistance to five (the above plus tetracycline) in one isolate: a ribotype 001, toxinotype 0 strain. Whilst ribotype 001 was the most commonly encountered type, there was no evidence of clonal relationships when all other typing and antibiotic resistance patterns were taken into account.

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INTRODUCTION

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus. It is commonly associated with a spectrum of disease referred to as *C. difficile*-associated disease (CDAD), which can range from uncomplicated mild diarrhoea to lethal toxic megacolon and possible colon perforation (Johnson & Gerding, 1998). It is considered to be the leading cause of nosocomially acquired diarrhoea in adults and can be responsible for large outbreaks (Kelly & LaMont, 1998). There is a view that the severity of the disease is increasing. The new hypervirulent type (ribotype 027, toxinotype III, pulse-field NAP1) in North America and several European countries has been associated with more severe and fatal cases (McDonald *et al.*, 2005; Kuijper *et al.*, 2006a; Hubert *et al.*, 2007).

As elsewhere, *C. difficile* is rarely cultured in Scotland and laboratory diagnosis depends on the detection of toxins A and/or B in faeces. Several phenotypic and molecular methods have been applied to determine the relatedness of strains of *C. difficile*. All have their advantages and disadvantages. Methods based on whole-genome analysis are more discriminatory, but they are technically demanding and labour-intensive (Brazier, 2001). PCR ribotyping is commonly used in Europe as it has been reported to be

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Abbreviation: CDAD, *Clostridium difficile*-associated disease.

highly discriminative, reproducible, relatively rapid and easy to perform (O'Neill *et al.*, 1996; Stubbs *et al.*, 1999). Toxinotyping is a PCR-RFLP method that depends on changes in the toxin genes and other regions of the pathogenicity locus of *C. difficile*. It has been reported to correlate well with restriction endonuclease analysis, serotyping and PCR ribotyping and it also gives the advantage of determining toxin variant strains (Rupnik *et al.*, 1998; Johnson *et al.*, 2003). Flagellin gene RFLP analysis has been described as an additional typing method that can be used in conjunction with other typing methods (Tasteyre *et al.*, 2000).

An actin-specific ADP-ribosylating binary toxin CDT is produced by some strains of *C. difficile*. Its role in pathogenesis is currently unclear, but its presence has been correlated with severity of disease in some studies (Barbut *et al.*, 2005) and it is present in the 027 hypervirulent strain (McDonald *et al.*, 2005). The prevalence of binary toxin in clinical isolates of *C. difficile* is generally low, with frequencies ranging between approximately 2 and 20 % (Barbut *et al.*, 2005).

The aims of this study were to (i) characterize 149 *C. difficile* isolates from toxin-positive faecal samples collected between August and October 2005 by molecular typing methods, PCR ribotyping, toxinotyping and flagellin gene RFLP analysis and by the S-layer typing method, together with the detection of the binary toxin genes *cdtA* and *cdtB*; and (ii) determine the susceptibility of isolates to seven different antibiotics. The objectives were to show which strains were currently present locally, to determine whether there were any clonal relationships between isolates and to examine the antimicrobial susceptibility profiles of the different types.

METHODS

Bacterial isolates. *C. difficile* isolates (n=149) from toxin-positive faecal samples (determined using a Toxin A+B ELISA kit; TechLab) were collected between August and October 2005 and stored at −20 °C. All specimens were from different unselected cases of CDAD in different hospitals in the Edinburgh area (Lothian University Hospitals National Health Service Trust) consisting of acute and long-stay hospitals. They were collected on a purely random basis with no selection for hospital or patient type. During this period, we were not aware of any outbreaks. Stool samples were cultured on Brazier's cefoxitin/cycloserine/egg yolk agar (LabM) and incubated for 48 h at 37 °C in an anaerobic chamber. The isolates were identified by characteristic colony morphology, smell, fluorescence under long-wave UV light and appearance on a Gram film. Subcultures were stored in anaerobic investigation medium containing cooked meat particles for maintenance (Brown *et al.*, 1996). Control strains were NCTC 11223, VPI 10463, 338a (a locally isolated strain of ribotype 01; McCoubrey, 2002), the sequenced strain 630 and a 027 strain from Amsterdam provided by E. Kuijper (Leiden, The Netherlands).

DNA extraction. Colonies from overnight anaerobic cultures on fastidious anaerobe agar (LabM) supplemented with 6 % horse blood were resuspended in 100 µl of a 5 % solution of Chelex-100 resin (Bio-Rad). After incubating in a boiling bath for 10 min, the cell debris was removed by centrifugation for 2 min at 18 000 ***g***. The supernatant was used as the crude DNA template for PCRs except for toxinotyping.

For toxinotyping, pure DNA isolation was required. DNA was extracted using a Nucleospin Tissue kit (Macherey-Nagel) according to the manufacturer's instructions.

PCR ribotyping. All 149 isolates were typed by PCR ribotyping according to the method described by O'Neill *et al.* (1996). Specific oligonucleotide primers 5′-CTGGGGTGAAGTCGTAACAAGG-3′ (nt 1445–1466 of the 16S rRNA gene) and 5′-GCGCCCTTTGTA-GCTTGACC-3′ (nt 20–1 of the 23S rRNA gene) complementary to the 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene were used to amplify the variable-length intergenic spacer region. The 338a and 027 strains were used as controls for ribotypes 001 and 027. Patterns that were different from these two ribotypes were compared with the library of PCR ribotypes already established at the Anaerobe Reference Unit, Cardiff, UK.

Toxinotyping. All 149 isolates were subjected to toxinotyping by the methods developed by Rupnik *et al.* (1997, 1998). The first 3 kb of *tcdB* (PCR fragment B1) and the 3 kb repetitive region of *tcdA* (PCR fragment A3) were detected and characterized by RFLP. The primers 5′-AGAAAATTTTATGAGTTTAGTTAATAGAAA-3′ and 5′-CAG-ATAATGTAGGAAGTAAGTCTATAG-3′ for the B1 fragment and 5′-TATTGATAGCACCTGATTTATATACAAG-3′ and 5′-TTATC-AAACATATATTTTAGCCATATATC-3′ for the A3 fragment were used as described by Rupnik *et al.* (1997). PCRs were performed in a final volume of 50 µl with a reaction mixture containing 20 mM Tris/HCl (pH 8.3), 50 mM KCl, 1 % W-1, 3 mM $MgCl_2$, 1 U *Taq* polymerase (Invitrogen), 200 µM each dNTP, 15 pmol each primer and 5 µl template DNA. For amplification of A3 fragments, tetramethylammonium chloride (Sigma) was added to a final concentration of 10^{-4} M. After initial denaturation at 93 °C for 3 min, B1 products were amplified for 30 cycles and A3 products for 35 cycles of annealing and extension at 47 °C for 8 min and denaturation at 93 °C for 4 s. Final extension was at 47 °C for 10 min. Amplified fragments were visualized on a 1 % agarose gel and subjected to restriction enzyme digestion using the restriction enzymes *Acc*I, *Hin*cII (B1) and *Eco*RI (A3). After electrophoresis of the digestion products, the toxinotypes of all tested isolates were determined using the toxinotyping schema described by Rupnik *et al.* (1997, 1998).

Detection of binary toxin genes. The presence of binary toxin genes among all 149 study isolates was detected by PCR as described by Stubbs *et al.* (2000). Primers designed to amplify the genes encoding the enzymic (*cdtA*) and binding (*cdtB*) components of the binary toxin were as follows: CDTA-F, 5′-TGAACCTGGAAAAGG-TGATG-3′; CDTA-R, 5′-AGGATTATTTACTGGACCATTTG-3′; CDTB-F, 5′-CTTAATGCAAGTAAATACTGAG-3′; CDTB-R, 5′-AACGGATCTCTTGCTTCAGTC-3′. The 027 strain, which is known to produce binary toxin, was used as the positive-control strain. The product sizes for *cdtA* and *cdtB* were 375 and 510 bp, respectively.

PCR-RFLP analysis of the flagellin (*fliC*) gene. The *fliC* gene of 147 of the study isolates (two were lost) was amplified using the specific primers Nter (5′-ATGAGAGTTAATACAAATGTAAGTGC-3′) and Cter (5′-CTATCCTAATAATTGTAAAACTCC-3′) corresponding to the 5′- and 3′-end sequences of the *fliC* gene of *C. difficile* (Tasteyre *et al.*, 2000). Amplification was carried out in a final volume of 50 µl reaction mixture containing 20 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 1 U *Taq* polymerase (Promega), 0.2 mM each dNTP, 1 mM each primer and 5 µl template DNA. Initial denaturation was carried out at 94 °C for 5 min, followed by

35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. A final step of extension for 10 min at 72 °C was performed. Products of 870 bp were digested with the restriction enzymes *Hpa*I, *Hin*dIII and *Rsa*I. Digested products were electrophoresed on a 1.2 % agarose gel to determine their RFLP groups. The restriction enzyme *Hin*cII was used for further differentiation between group I and group III flagella types.

S-layer typing. The S-layer typing of *C. difficile* isolates was performed as described previously (McCoubrey *et al.*, 2003). Briefly, the isolates were subcultured and S-layer proteins were extracted with 5 M guanidine hydrochloride. The resulting two major and several minor bands were visualized by SDS-PAGE (Invitrogen) with Coomassie staining. Mark 12 molecular mass standards were used as calibrations for the calculation of molecular masses. Banding patterns were compared with the previous types.

Antibiotic susceptibility testing. The MICs of 116 of the isolates for six antibiotics were determined using the agar dilution protocol in the NCCLS guidelines (NCCLS, 2001). The antibiotics and concentrations used were as follows: 16–0.125 μg ml^{-1} for vancomycin, 32–0.5 μg ml^{-1} for metronidazole, 256–4 μg ml^{-1} for ceftriaxone, 32–0.5 μg ml^{-1} for clindamycin, 32–0.5 μg ml^{-1} for erythromycin and 64–1 μg ml^{-1} for tetracycline (all from Sigma). The isolates were subcultured from cooked meat broth into pre-reduced thioglycollate medium (Sigma) enriched with 5 μg haemin, 1 μg vitamin K_1 and 1 mg $NaHCO_3$ ml^{-1} and incubated overnight in an anaerobic chamber at 37 °C. After adjusting the turbidity to a 0.5 McFarland standard, aliquots (1–2 μl) of the cultures were spotted onto *Brucella* agar (Oxoid) supplemented with haemin, vitamin K_1 and 5 % lysed horse blood plus antibiotic of a given concentration using a multipoint inoculator and incubated anaerobically at 37 °C for 48 h. Control plates were also inoculated and incubated aerobically to check for aerobic growth. Strains NCTC 11223, 338a and 630 were used as control strains as their MICs were known from our previous study (Drummond *et al.*, 2003).

The MICs of the isolates for moxifloxacin were determined by the Etest (AB Biodisk) as we were unable to obtain the pure substance from Bayer. The isolates were grown in pre-reduced thioglycollate medium (Sigma) enriched with 5 μg haemin, 1 μg vitamin K_1 and 1 mg $NaHCO_3$ ml^{-1} and incubated overnight in an anaerobic chamber at 37 °C. The Etest was carried out by inoculating the surface of pre-reduced fastidious anaerobe agar (LabM) plates containing vitamin K_1, haemin and 5 % lysed horse blood with a 1 McFarland standard-matched inoculum. The inoculation was performed with cotton-tipped swabs and Etest strips were applied to the agar surface according to the manufacturer's instructions. Sufficient growth was obtained after 24 h and the ellipse was clearly visible. The end points were read at complete inhibition of all growth, including hazes and isolated colonies. Strains 630 and 027 were used as sensitive and resistant controls, respectively.

Breakpoints of susceptibility for each drug were chosen at the levels listed by the NCCLS. MIC_{50} and MIC_{90} values for each isolate were calculated using Microsoft EXCEL.

RESULTS AND DISCUSSION

Molecular typing

The 149 isolates included in this study were collected over a period of 2 months between August and October 2005. The results of the different typing methods are summarized in Table 1. All of the isolates were typable by the PCR ribotyping method and 15 different ribotype patterns could be discriminated. Ribotype 001 was the most common (n=113, 75.8 %), followed by ribotype 106 with 12 isolates (8.1 %). The other ribotypes identified were 005 and 014 with four isolates each, ribotype 002 with three isolates and ribotypes 013 and 126 with two isolates, whilst the other ribotypes (020, 023, 042, 049, 070, 171) were represented by single isolates. Three isolates belonging to two different ribotypes were not able to be allocated a specific ribotype and may represent new types. No 027 strain was found. However, during the preparation of this manuscript the first case of 027 in Scotland was reported from the Glasgow area. These findings are consistent with other reports from

Table 1. Numbers and types of isolates determined by PCR ribotyping, toxinotyping and *fliC* restriction analysis

Ribotype (no. detected out of 149)	Different toxinotypes found for each ribotype					Different *fliC* patterns for each ribotype	
	0	I	IV	V	XIII	I	VII
001 (113)*	111	1	–	–	1	–	112
106 (12)*	12	–	–	–	–	1	10
005 (4)	4	–	–	–	–	–	4
014 (4)	4	–	–	–	–	–	4
002 (3)	3	–	–	–	–	–	3
013 (2)	2	–	–	–	–	–	2
126 (2)	–	–	–	2	–	–	2
020 (1)	1	–	–	–	–	–	1
023 (1)	–	–	1	–	–	–	1
042 (1)	1	–	–	–	–	1	–
049 (1)	1	–	–	–	–	–	1
070 (1)	1	–	–	–	–	–	1
171 (1)	1	–	–	–	–	–	1
Others (3)	3	–	–	–	–	–	3

*One isolate from each of these ribotypes could not be typed by *fliC* restriction analysis.

the UK prior to the recognition of the 027 strains in several areas of England. PCR ribotype 001 has been reported to be the most common type (55 %) among hospitalized patients in the UK (Stubbs *et al.*, 1999). In an earlier study by our group, ribotype 001 was responsible for 78 % of CDAD infections locally (McCoubrey *et al.*, 2003). Recently, ribotype 106 has become prominent in England. In the period 1995–2003 it was at similar levels to those found in our study (8 %); however, in 2005 it was the predominant strain at 26 %, just above 027 strains and surpassing the 001 strains, which were both at 25 % (Health Protection Agency, 2006). Prior to the recognition of 027 strains in Europe, particularly in The Netherlands and Belgium, there are only a few reports from other countries in Europe. In a Polish hospital, all environmental isolates and 11 of 31 neonatal isolates were found to belong to ribotype 001 (Martirosian *et al.*, 1995), whereas ribotype 087 accounted for 39 % of all isolates in Hungary (Urban *et al.*, 2001). In one report from the Middle East, ribotypes 097 and 078 were reported to be responsible for over one-third of the cases of CDAD in Kuwaiti hospitals (Rotimi *et al.*, 2003).

All of the isolates were subjected to toxinotyping. The B1 and A3 fragments of *tcdA* and *tcdB* were amplified as they have been reported to be the most variable fragments and good markers when searching for variant strains (Rupnik, 2001). *C. difficile* VPI 10463 (which has been defined as toxinotype 0; Rupnik *et al.*, 1998) was used as a reference strain. This toxinotype 0 was observed in the majority of the isolates (96.6 %, n=144). The five remaining isolates were of four different known toxinotypes: toxinotypes I, IV and XIII with one isolate each and toxinotype V with two isolates. No toxinotype III strains were found. There was no previous local information on the prevalence and distribution of different toxinotypes. When compared with the studies from Europe, the rates of variant (not 0) toxinotypes in our study were lower (Rupnik *et al.*, 1998, 2001; Spigaglia & Mastrantonio, 2002). The profiles of the strain collections in these studies were different but non-toxigenic strains were not included in our study. The prevalence of variant toxinotypes has been reported to be 21.5 % among the selected *C. difficile* isolates from 22 serogroups tested by Rupnik *et al.* (1998) and was estimated for the Cardiff collection as 8.8 % among the toxinogenic strains (Rupnik *et al.*, 2001). The percentage of variant strains from Asia has been reported to be 23.5 %, whilst 25 % of the toxinogenic strains from Italy were found to show variation (Spigaglia & Mastrantonio, 2002; Rupnik *et al.*, 2003a). Of the strains from an American hospital, 11.1 % belonged to variant toxinotypes. The most frequent variant toxinotypes in two European collections were toxinotypes III, IV and VIII (Rupnik *et al.*, 1998, 2001). Our variant strains were of toxinotypes I, IV, V and XIII. The toxinotype IV strain was of ribotype 023, whilst two toxinotype V strains were of ribotype 126. Toxinotypes I and XIII were of ribotype 001. In our study, among the 15 ribotypes that were determined, all isolates within a ribotype except ribotype 001 belonged to a single toxinotype. This was similar to the findings of Rupnik *et al.* (2001) where PCR ribotyping and toxinotyping were shown to correlate well. In the case of different toxinotypes within a PCR ribotype, these toxinotype profiles were found to be similar. In our study, only five isolates of toxinotypes other than 0 were found and isolates of ribotype 001 belonged to three different toxinotypes. Toxinotype I and XIII strains have been reported to differ from toxinotype 0 only at the 3′ end of the *tcdA* gene (Rupnik *et al.*, 1998).

PCR amplification of the *fliC* gene from 147 isolates produced an 870 bp fragment. Two different restriction profiles were obtained when the amplification products were digested using the enzymes *Hpa*I, *Hin*dIII and *Rsa*I. All isolates but two were of restriction pattern VII, the exceptions being restriction pattern I, and were of ribotypes other than 001 (ribotypes 106 and 042) but were of toxinotype 0. Tasteyre *et al.* (2000) compared PCR-RFLP analysis of the flagellin gene and serogroups in a collection of strains representing all of the 12 known serotypes from widely different geographic areas. They reported that this method could constitute an additional typing method to be used in conjunction with other methods. They found RFLP type VII as the most frequent RFLP type, followed by types I and VIII. RFLP type VII strains were mostly toxin-positive strains, whereas type I strains were either toxin positive or negative. They found that RFLP types II, III, IV, V and VI were uncommon and only associated with single serogroups. Our study is the first to compare ribotyping and toxinotyping with flagellin gene typing. However, only two flagella types were detected (I and VII): type VII strains contained different ribotypes and toxinotypes and type I strains were of different ribotypes but all of toxinotype 0. Thus a larger number of strains from different ribotypes and toxinotypes are needed to be able to determine the relationships between these typing methods.

All strains (n=149) were tested for the presence of binary toxin genes (*cdtA* and *cdtB*), but only three (2 %) harboured these genes. Similar to the number of variant strains encountered above, the presence of binary toxin genes was low compared with other studies. It has been reported that 6.4 % of toxigenic isolates of *C. difficile* referred to the Anaerobe Reference Unit from UK hospitals had both binary toxin genes (Stubbs *et al.*, 2000) and 4.5, 5.8 and 8.6 % prevalence of binary toxin-positive strains was detected in Spain, America and Poland, respectively (Geric *et al.*, 2004; Alonso *et al.*, 2005; Pituch *et al.*, 2005). Pituch *et al.* (2005) found that all binary toxin-positive strains from Poland were of the same toxinotype, type IV, and were all of the same ribotype. In our study, two of the binary toxin-positive isolates were of ribotype 126, toxinotype V, and one isolate belonged to ribotype 023, toxinotype IV. In most studies, it has been shown that only strains belonging to variant toxinotypes that have significant changes in *tcdA* and *tcdB* possess binary toxin genes (Stubbs *et al.*, 2000; Rupnik *et al.*, 2003b). Geric *et al.*

(2003) reported A^-B^- strains with binary toxin genes. We had only toxigenic strains in our study. Our strains of toxinotypes IV and V with changes in both the *tcdA* and *tcdB* genes had binary toxin genes, whereas strains of toxinotypes I and XIII with minor differences only in the 3′ end of the *tcdA* gene did not have *cdtA* or *cdtB*.

S-layer typing

In the past, we have used S-typing as our primary method for epidemiological studies of *C. difficile* (McCoubrey *et al.*, 2003). We were interested in correlating the different molecular types with S-type. However, as it is relatively labour-intensive, we selected a sample of only 62 isolates to represent the range of molecular types described above with the result that six different S-types were recognized. Most isolates (64.5 %; $n=40$) belonged to the common S-type, known as type 4939, named for the molecular masses of the two S-layer peptides. In an earlier paper (McCoubrey *et al.*, 2003), this common S-type, which is that of ribotype 001, was referred to as 5336. We have recently changed our SDS-PAGE system to a commercial system (Invitrogen) employing 10 % gels. With this new method, the molecular masses of the S-layer proteins are found to be different. Most of the others were of S-types 4741 (21 %; $n=13$) and 4640 and 4938 with three isolates each (4.8 %). Of the remainder, two isolates (3.2 %) were of S-type 4639 and one isolate (1.6 %) was of S-type 4837. All ribotype 001 isolates ($n=37$) were of the same S-type (4939), whilst three of the isolates from different ribotypes were also of this S-type. All but one of the ribotype 106 isolates ($n=11$) were of S-type 5242, the other being of the common 4939 type. Ribotypes 002 and 014 also contained different S-types. The toxinotype 0 isolates ($n=59$) belonged to all six S-types, with the most common being S-type 4939 ($n=38$). Toxinotype I and XIII isolates, which were also of ribotype 001, were of S-type 4939.

The discriminatory power of different typing methods is an important consideration when selecting which method to use. The usual gold standard of PFGE is generally considered to have a higher degree of discrimination than PCR ribotyping. However, in the past, the typing ability of PCR ribotyping was higher than that of PFGE because DNA degradation occurred as a result of endogenous restriction enzymes in strains from serogroup G, which corresponds to PCR ribotype 001 (Collier *et al.*, 1996; Bidet *et al.*, 2000).

Currently, PCR ribotyping is preferred because of the ease and speed of the technique and because it is reported to be highly discriminatory and reproducible. Toxinotypes are reported to correlate well with the types obtained by two other typing schemes, serogrouping and PFGE typing (Rupnik *et al.*, 1998), whilst toxinotyping and ribotyping methods correlate well. Most strains within a PCR ribotype belonged to a single toxinotype. Strains in toxinotypes I, III, IV, VI and VIII could be differentiated into several PCR ribotypes (Rupnik *et al.*, 2001).

Antibiotic susceptibility testing

A major aim of this study was to determine the current antibiotic susceptibility patterns of the *C. difficile* isolates in our region and to find out whether there was any relationship between the types and antibiotic susceptibilities. The susceptibility to antibiotics was investigated in a sample of 116 isolates of our collection by determining the MICs for seven antibiotics: metronidazole, vancomycin, erythromycin, clindamycin, ceftriaxone, moxifloxacin and tetracycline. Table 2 shows the ranges of MICs and resistance rates among the isolates for the seven antibiotics used, together with MIC_{50} and MIC_{90} values and breakpoints for the antibiotics. All isolates were sensitive to the two agents commonly used to treat CDAD, metronidazole and vancomycin, with a narrow range of MICs.

In our previous study (Drummond *et al.*, 2003), no resistance to metronidazole or vancomycin was reported. MIC ranges and MIC_{50} and MIC_{90} values for these antibiotics were similar to those in the present study. However, the number of isolates with an MIC of 4 μg ml^{-1} for vancomycin increased from 5 out of 186 isolates (2.7 %) in our earlier study to 25 out of 116 isolates (21.6 %) in the present study. Vancomycin and metronidazole are the most common antibiotics used in the treatment of CDAD and, in most studies, isolates of *C. difficile* have generally been found to be susceptible to these (Drummond *et al.*, 2003; Aspevall *et al.*, 2006). However, a few studies have reported strains resistant to metronidazole or with reduced susceptibility to vancomycin (Brazier *et al.*,

Table 2. Range of MIC values and resistance rates from 116 isolates with the breakpoints used

Antibiotic	MIC range (μg ml^{-1})	MIC_{50} (μg ml^{-1})	MIC_{90} (μg ml^{-1})	Breakpoint (μg ml^{-1})	Resistance (%)
Vancomycin	1–4	2	4	⩾8	0
Metronidazole	⩽0.5–4	1	2	⩾8	0
Erythromycin	0.5–⩾32	⩾32	⩾32	⩾8	94.8
Clindamycin	0.5–⩾32	8	16	⩾8	62.9
Ceftriaxone	32–256	64	64	⩾64	87.1
Moxifloxacin	0.25–⩾32	⩾32	⩾32	⩾4	87.1
Tetracycline	⩽1–64	⩽1	2	⩾16	4.3

2001; Peláez *et al.*, 2005). The first UK isolate of *C. difficile* with reduced susceptibility to metronidazole was reported in 2001 (Brazier *et al.*, 2001).

Resistance to clindamycin was seen in 73 isolates (62.9 %). Tetracycline resistance was low, with only five isolates with MICs $\geqslant$16 μg ml^{-1}. MIC_{50} and MIC_{90} values for erythromycin were $\geqslant$32 μg ml^{-1}, showing that the majority of the isolates were highly resistant to this antibiotic (n=110, 94.8 %). Similar high resistance rates to the antibiotics ceftriaxone and moxifloxacin (87.1 %) were also found. Their MIC_{50} values were high and the same as their MIC_{90} values.

Previously, moxifloxacin was reported to have good activity against Gram-positive bacilli including *C. difficile* (Hoogkamp-Korstanje & Roelofs-Willemse, 2000), but reduced susceptibility to this antibiotic has been shown in several studies (Wilcox *et al.*, 2000; Leroi *et al.*, 2002). Clindamycin and ceftriaxone resistance rates did not show much difference from our previous study (Drummond *et al.*, 2003).

Antibiotic susceptibility in relation to molecular type

Of the 116 isolates for which MICs were measured, 87 were of ribotype 001, 10 were of ribotype 106 and 19 were of other ribotypes. In terms of toxinotype, 112 were of toxinotype 0, with one isolate each of toxinotypes I, IV, V and XIII. The antibiotic resistance patterns of these 116 isolates are detailed in Table 3.

In a study from the UK, PCR ribotypes 001 and 106 were found to be more resistant to erythromycin (98 and 100 %, respectively) than other PCR ribotypes (John & Brazier, 2005). All of our ribotype 001 and 106 ribotypes were resistant to this antibiotic. We also found higher resistance levels to the antibiotics ceftriaxone and moxifloxacin, which were not tested in that study, among ribotype 001 and 106 isolates than among the other ribotypes. John & Brazier (2005) reported that clindamycin resistance was lower than erythromycin resistance in ribotypes 001 and 106, whilst ribotypes 015, 014, 005 and 002 had a higher frequency of resistance to clindamycin than to erythromycin. Of our isolates, only ribotype 002 had a higher clindamycin resistance level than erythromycin, but the frequency of clindamycin resistance was lower for ribotype 014, whilst both resistance rates were the same for ribotype 005. In a study in which clindamycin and fusidic acid resistances were determined (Aspevall *et al.*, 2006), no particular relationship between PCR ribotypes and antibiotic resistance was found. The clindamycin resistance frequency in our study was lower than that found by Aspevall *et al.* (2006) (83 %). In a study from Australia, the MIC range for moxifloxacin (0.75 to >32 μg ml^{-1}) was found to be close to the resistance breakpoint with MIC_{50} and MIC_{90} values of 2 and 4 μg ml^{-1}, respectively (Leroi *et al.*, 2002). Susceptibilities of clonal and distinct *C. difficile* strains from the UK to newer fluoroquinolones including moxifloxacin have been tested (Wilcox *et al.*, 2000). Trovafloxacin and moxifloxacin were the most active fluoroquinolones with three- to fourfold more activity than older agents such as ciprofloxacin among genotypically distinct strains. Clonal strains that were epidemic ribotype 001 strains were sevenfold less susceptible to moxifloxacin compared with the distinct strains. The MIC range for this antibiotic was 0.12–16 μg ml^{-1} and MIC_{50} and MIC_{90} values were 1 and 16 μg ml^{-1}, respectively (Wilcox *et al.*, 2000). We found higher MIC_{50} and MIC_{90} values for moxifloxacin in our study. Most of the ribotype 001 (98.9 %, n=86) and ribotype 106 (90 %, n=9) isolates were resistant to moxifloxacin, whereas only six isolates (31.5 %) from other ribotypes were resistant in our study.

Only two isolates of flagellin gene restriction pattern I were observed. All of these restriction type I isolates were sensitive to tetracycline and moxifloxacin and resistant to erythromycin. One was resistant to both clindamycin and

Table 3. Percentage antibiotic resistance rates by ribotype and toxinotype

Antibiotic	Percentage of isolates resistant to antibiotics based on:							
	Ribotype			Toxinotype				
	001 (n=87)	106 (n=10)	Other (n=19)	0 (n=112)	I (n=1)*	IV (n=1)*	V (n=1)*	XIII (n=1)*
Erythromycin	100	100	68.4	95	R	S	R	R
Clindamycin	62.1	70	63.1	62.5	R	S	R	R
Ceftriaxone	95.4	100	42.1	87.5	R	R	S	R
Moxifloxacin	98.9	90	31.5	88.3	R	S	S	R
Tetracycline	1.2	0	21.1	3.57	S	S	R	S
Metronidazole	0	0	0	0	S	S	S	S
Vancomycin	0	0	0	0	S	S	S	S

*As toxinotypes I, IV, V and XIII are represented by single isolates, the designations R (resistant) and S (sensitive) have been used rather than percentages.

ceftriaxone, whilst the other was sensitive to both antibiotics.

One of the two isolates carrying the binary toxin genes was resistant to erythromycin, clindamycin and tetracycline, whilst the other one was only resistant to ceftriaxone. Both isolates were sensitive to moxifloxacin.

Fifty-six of the 62 isolates that were typed by S-layer were tested for antibiotic susceptibility. Most of the S-type 4939 isolates (38/39) and all of the S-type 4741, 4938 and 4639 isolates (n=10, n=3 and n=2, respectively) were resistant to erythromycin. Most of the S-type 4939 and 4741 isolates were resistant to clindamycin, ceftriaxone and moxifloxacin. One isolate that belonged to S-type 4837 was sensitive to all antibiotics. This isolate was of ribotype 070, toxinotype 0 and *fliC* restriction type VII.

Multi-resistant strains

Seventy-two isolates were resistant to both erythromycin and clindamycin in our study, with resistance to both antibiotics being 62, 70 and 52.6 % in ribotypes 001, 106 and the others, respectively. Of toxinotype 0 and other toxinotype isolates, 61.6 and 75 % were resistant to both antibiotics, respectively. Only one *fliC* restriction pattern I isolate and one binary toxin gene-positive isolate resistant to these antibiotics were encountered. Macrolide–lincosamide–streptogramin B resistance in *C. difficile* is mostly encoded by the *ermB* resistance determinant. This gene encodes a 23S rRNA methyltransferase that modifies the target site for the antibiotic and is a mobilizable, conjugative transposon, Tn*5398* (Farrow *et al.*, 2001). In recent years, some *ermB*-negative isolates with erythromycin and clindamycin resistance have been reported (Ackermann *et al.*, 2003; Spigaglia & Mastrantonio, 2004; Pituch *et al.*, 2006). Spigaglia & Mastrantonio (2004) could not find any *erm* genes of other classes such as *ermA*, *ermC*, *ermF*, *ermQ* and *mefA* among these isolates. It has been suggested that resistance in *ermB*-negative resistant strains could be due to mutations within the target sequences in the 23S rRNA or efflux mechanisms or a new mechanism of resistance (Ackermann *et al.*, 2003; Spigaglia & Mastrantonio, 2004; Pituch *et al.*, 2006). We did not test our isolates for resistance genotypically.

A total of 66 isolates were resistant to the three antibiotics erythromycin, clindamycin and ceftriaxone and 96 isolates were resistant to erythromycin, ceftriaxone and moxifloxacin. Ribotypes 001 and 106 had higher resistances (95.4 and 90 %, respectively) to the antibiotics erythromycin, ceftriaxone and moxifloxacin when compared with other PCR ribotype groups (21 %). Ackermann *et al.* (2001) suggested that resistance to moxifloxacin might be due to amino acid substitution in the DNA gyrase. They also found that moxifloxacin-resistant strains that were selected *in vitro* had wild-type *gyrA* sequences.

In our study, only one isolate was resistant to five antibiotics: erythromycin, clindamycin, moxifloxacin, ceftriaxone and tetracycline; it was of ribotype 001 and toxinotype 0. Ackermann *et al.* (2003) reported resistances to the antibiotics erythromycin, clindamycin and moxifloxacin as 27, 36 and 12 %, respectively, among 192 isolates tested. They found that moxifloxacin resistance was almost always detected together with resistance to erythromycin and clindamycin (12.5 %). In our study, 110 erythromycin-resistant isolates were found of which 100 (90.9 %) were resistant to moxifloxacin and only one out of six erythromycin-sensitive isolates was resistant to moxifloxacin. Among the 73 clindamycin-resistant isolates, 64 (87.7 %) were also resistant to moxifloxacin. Of the 72 isolates resistant to both erythromycin and clindamycin, 64 (88.9 %) were resistant to moxifloxacin. Additionally, we found that all but one of the 64 isolates that were resistant to these three antibiotics were also resistant to ceftriaxone and these multi-resistant isolates were mostly of ribotype 001 (n=53, 84.1 %).

As the 027 type has a characteristic antibiotic resistance pattern – resistant to erythromycin, susceptible to clindamycin and resistant to moxifloxacin (Kuijper *et al.*, 2006b) – this could be part of an algorithm to identify 027 strains. However, in our study we identified 36 strains that were resistant to erythromycin, susceptible to clindamycin and resistant to moxifloxacin. Thirty-two of these strains were of ribotype 001, three were of ribotype 106 and one was of 014, and all of them were of toxinotype 0. This questions the usefulness of this approach to detect 027 strains.

We are aware that a Europe-wide surveillance study has been performed (F. Barbut and others, unpublished) and some strains for this study were collected in Scotland. However, there was no overlap in strains between these studies as those for the European surveillance study were collected earlier in 2005.

The results obtained from this study demonstrate clearly the complexity of the strains of *C. difficile* in our area. If characterized purely on ribotyping, it would appear that most of the strains are closely related. However, the use of other typing methods, especially antibiotic resistance patterns, demonstrates wide variation among strains.

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